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# Carboxymethylcellulose as a Carrier for Follicle Stimulating Hormone in the Female Rat and Bovine

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# CARBOXYMETHYLCELLULOSE AS A CARRIER FOR FOLLICLE STIMULATING HORMONE IN THE FEMALE RAT AND BOVINE

BY

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FEMALE RAT AND BOVINE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

# CARBOXYMETHYLCELLULOSE AS A CARRIER FOR FOLLICLE STIMULATING HORMONE IN THE FEMALE RAT AND BOVINE

Abstract

C. LEROY JOHNSON

Under the supervision of Assistant Professor T. D. Rich
and Professor C. A. Dinkel

A series of biological assays were conducted to compare physiological saline (PSS) and carboxymethylcellulose (CMC) as carriers for follicle stimulating hormone (FSH) and to determine the optimum ratio of FSH to CMC. Constant individual dosages of 0.5 mg FSH were injected into a total of 384 immature female rats in four experiments. Treatments included single injections of FSH in PSS, FSH in 1 percent CMC at ratios (mg FSH:ml CMC) of 1:3, 1:2, 1:1, 2:1, 3:1, 5:1 and FSH in 2 percent CMC at a ratio of 1:1. Parameters of primary and secondary response were ovarian and uterine weight, respectively, at 24, 48, 72 and 96 hours post-injection.

It was concluded that ovarian and uterine growth resulting from FSH in CMC was greater than from the same quantity of FSH in PSS. The apparent optimum ratio of FSH to CMC was 1 mg FSH in 2 ml or more of 1 percent CMC. The mechanisms by which CMC tended to enhance the activity of FSH could not be determined from the data.

A total of 99 yearling, commercial beef heifers was used in two experiments conducted during consecutive summers (1970 and 1971) to study ovarian response to FSH carried in CMC. Each trial was conducted in two phases to provide two data collections from each group of experimental animals.

In the first experiment, all heifers were fed 1 mg melengesterol acetate (MGA) per head per day for 14 days to synchronize estrus. Seven mg FSH in 1 percent CMC injected on days 10, 12 or 14 of MGA feeding did not affect ovarian stimulation (ovulation sites or ovulation sites plus follicles $\geq$10 mm) significantly. Five mg estradiol valerate (EV) injected on day 2 of MGA feeding to induce regression of luteal tissue tended to reduce ovulation rate ($P<.10$) in response to exogenous FSH. However, a significant phase x EV x day of injection interaction ($P<.01$) limited positive conclusions relative to this effect. The incidence of anovulatory heifers was relatively high (8/48 and 8/49 in phases I and II, respectively) and conception rates from a single artificial insemination at post-treatment estrus in phase II was low for all groups (0 to 29 percent).

In phase I of the second experiment, total doses of 7 or 10 mg FSH in CMC (1 mg FSH:2 ml 1 percent CMC) injected as single doses on day 15 or two half-doses on days 15 and 17 of natural estrous cycles were not significant sources of variation in ovarian stimulation. In phase II, ovarian stimulation was not affected significantly by 5 mg injections of FSH on days 15 and 17 or on days 16 and 18 (total of 10 mg) of natural estrous cycles or by the same treatments repeated to one-half of each group during one subsequent cycle. Five twin fetuses and nine nongravid uteri were observed in 45 heifers artificially inseminated during two estrous cycles.

Data from both experiments suggested that shorter intervals from FSH injection to estrus resulted in greater ovarian stimulation.

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# GENERAL INTRODUCTION

Economic gain is the ultimate goal of the beef cattle producer. Since costs associated with the maintenance of a beef cow herd are relatively fixed in given management situations, net return is directly related to reproductive performance, provided it is not achieved at the expense of livability, growability or desired carcass traits. The production of twin calves suggests definite economic advantages to cow-calf operators, especially to those operating relatively intensive production systems.

Natural twinning in cattle is considered infrequent and not highly heritable, but the possibility of increasing the incidence of twinning by treatment with exogenous gonadotropins has been recognized for nearly 30 years. However, the production of twin calves by this method has not been achieved on a commercial basis, largely because the limitation of ovarian stimulation to two viable ova per cow per estrus has persisted as a major obstacle.

Immediate problems in controlling ovulation rate are the selection of the hormone(s), the dosage(s), the time(s) of administration, the number of doses, suitable carrier(s) and their interrelationships.

The purposes of this study were twofold: (1) To study carboxymethylcellulose (CMC) as a carrier for follicle stimulating hormone (FSH) and (2) To search for a practical FSH-dose-time regime that might increase the incidence of twin births in beef cattle.

# GENERAL LITERATURE REVIEW

The induction of above normal ovulations through the use of exogenous gonadotropins has been well documented for several species. However, a practical hormone-dose-time technique has yet to be developed which will result in controlled ovulation rate of two ova per animal per estrus in beef cattle.

The following review is relative to the physiological behavior of follicle stimulating substances, primarily follicle stimulating hormone (FSH), carriers that have been used in their administration and methods for evaluating their biological activities. A review of several aspects considered in previous attempts to achieve controlled multiple ovulations in beef cattle is included.

## Follicle Stimulating Hormone

### Historical

The gonadotropic properties of whole or specific components of pituitary glands were first demonstrated by transplanting these tissues into laboratory animals. Smith (1926) reported the anterior lobe of the pituitary induced premature sexual maturity in hypophysectomized female rats. Also, daily pituitary transplants into adult, intact female rats resulted in an increase in size of ovarian follicles which frequently became cystic. Similarly, two mouse pituitaries were transplanted daily into adult, intact female mice commencing on the first day of estrus (Engle, 1927). Mating occurred within 12 hours after the second transplant and the number of uterine

implants observed at the ninth and tenth days of pregnancy ranged from 19 to 29. Immature mice implanted with anterior pituitary tissue exhibited precocious sexual maturity with appearance of the vaginal orifice followed by estrus in 1 to 5 days (Engle, 1931). In the studies cited, no attempts were made to identify the substance(s) responsible for the observed behavior of transplanted pituitary tissue.

Smith and Engle (1927) implanted fresh (1 hour or less old) mouse, rat, cat and guinea pig anterior pituitaries into immature mice and rats. Precocious sexual maturity was induced rapidly and ovarian weights of treated animals exceeded controls by tenfold. Transplants to adult female mice resulted in superovulation and the follicle was defined as the site of stimulatory action. Fevald, Hisaw and Leonard (1931) presented definite evidence for the presence of two distinct gonadotropic hormones in the anterior lobe of the pituitary. They used an aqueous pyridine extraction procedure and described the water soluble fraction as a stimulator of follicular growth. The insoluble fraction was described as luteinizing hormone, a stimulator of luteal growth. Fevald et al. (1933) verified the conclusions of earlier work that the anterior lobe contained two separate gonadotropic substances. One fraction developed follicles and the other aided in the formation of corpora lutea from previously developed follicles in immature rats and rabbits.

## Extraction and Purification

Subsequent workers developed methods of extracting and purifying those substances which were designated first as gonadotropins and

later, more specifically, as follicle stimulating hormone (FSH) and luteinizing hormone (LH). Criteria for evaluating purified gonadotropic extracts were suggested as follows (Greep, van Dyke and Chow, 1940):

1. Separation in single stages without serious loss.
2. Biological potency and purity established in hypophysectomized, immature rats.
3. Physiological and chemical evidence of near purity.

Fevald et al. (1931, 1933) used aqueous pyridine to extract two gonadotropins from desiccated sheep pituitary powder without injury to biological activity. Step-by-step procedures for extraction and purification of FSH from pituitaries of various domestic animals have been presented by Greep et al. (1940); Li, Simpson and Evans (1949); Steelman et al. (1953); McShan, Kagawa and Meyer (1954) and Leonora, McShan and Meyer (1956). The procedures of Steelman et al. (1953) have persisted as the most favorable in the preparation of commercial FSH (FSH-P, Armour-Baldwin Company, 1969).

Several procedures have been used to increase the purity of FSH. Enzymatic digestion of pituitary extracts prepared by the method of Steelman et al. (1953) followed by fractionation with alkaline and acid ethanol increased follicle stimulating activity 10 to 11 times the Armour Standard, 264-151X (Steelman, Lamont and Baltes, 1955). Synthetic cation and anion exchange resins have also been used (McShan et al., 1954; Raacke and Li, 1957).

Ion exchangers derived from cellulose, diethylaminoethyl-cellulose (DEAE cellulose) and carboxymethylcellulose (CMC) have been used to increase the purity and activity of pituitary extracts by the selective removal of inactive proteins and LH (Steelman et al., 1956; Ellis, 1958; Ward, McGregor and Griffin, 1959; Cahill, Payne and Shetlar, 1961).

Incomplete separation of LH from FSH was reported by Fevald et al. (1933). Since then, nonspecific terminology, apparently homogeneous, relatively pure and nearly pure, have been used by Greep et al. (1940), Li et al. (1949), Ellis (1958) and Cahill et al. (1961) to characterize highly active preparations. There has been general agreement among various workers that FSH, free of LH, has not yet been prepared. Slight but persistent LH activities in purified FSH fractions have been noted by Chen and van Dyke (1939); van Dyke, P'an and Shedlovsky (1950); Steelman et al. (1953, 1955) and Reichert and Parlow (1963). Steelman et al. (1956) reported FSH preparations with activities of 50 times standard retained a small LH activity. It was concluded that either the LH remaining after separation with ion exchangers has different properties from the LH held by the exchanger or it is inherent in the FSH molecule. In agreement, Woods and Simpson (1960) reported LH contamination in FSH that had 65 times the activity of Armour Standard.

## Properties

FSH is a relatively large molecule with an estimated molecular weight of approximately 29,000 (Steelman et al., 1956; Steelman and

Segaloff, 1959). Geschwind (1964) and Turner (1966) reported estimates of the molecular weight of FSH range from 19,000 to 67,000. The apparent isoelectric point of swine FSH is 5.1 to 5.2 (Steelman and Segaloff, 1959) or 4.5 and 4.8 for swine and sheep extracts, respectively (van Dyke et al., 1950). Steelman, Segaloff and Anderson (1959) reported FSH is more acidic than LH. Discrepancies between reports by different workers are apparently due to species differences and extent of purification of materials studied.

The chemical structure of FSH has not been described in detail. However, Steelman and Segaloff (1959) reported that swine FSH contained 80.7 percent amino acid and 7.4 percent carbohydrate. Gottschalk, Whitten and Graham (1960) demonstrated the presence of sialic acid in the molecule.

Bogdanove and Gay (1969) reported the half-life of FSH observed in rats ranged from 96 to 239 minutes with an average of 149 minutes but that exogenous FSH disappeared more rapidly than native FSH. The half-life of exogenous FSH in hypophysectomized rats ranged from 48 to 93 minutes. Parlow and Reichert (1963) suggested the biological half-life of porcine FSH may be more rapid than that of FSH from other sources. The apparent half-life of FSH may be much shorter than that of pregnant mare serum (PMS) which has been estimated at 6.00 to 6.55 days in hysterectomized shetland ponies (Cole et al., 1967). On the other hand, Catchpole, Cole and Pearson (1935) noted the rate of disappearance of mare gonadotropin in the rabbit was one-half every 26 hours and in the gelding one-half the first 72 hours followed by regular decreases of one-half every 6 days.

The persistence of FSH activity in the presence of proteolytic enzymes has been demonstrated by digestion of pituitary extracts with trypsin (McShan and Meyer, 1938; Chen and van Dyke, 1939). Chow, Greep and van Dyke (1939) demonstrated that digestion of pituitary extracts with pancreatin destroyed LH but not FSH. Alcohol fractionation after pancreatin digestion resulted in a FSH product 10 to 12 times as active as standard (Steelman et al., 1955). This material was not dialyzable; therefore, it was concluded that FSH is not a small active molecule attached to a large molecular weight protein.

The destruction of FSH activity by ptylin, high in amylolytic activity, has been demonstrated by McShan and Meyer (1938). The same workers (McShan and Meyer, 1940) reaffirmed the destructive action of ptylin and also demonstrated the inactivation of FSH by cysteine. They concluded FSH activity is associated with the carbohydrate portion of the molecule and that factors other than carbohydrates are necessary for activity.

In more recent work, Gottschalk et al. (1960) reported that treatment with a-neuraminidase (influenza virus enzyme and receptor destroying enzyme) resulted in enzymatic release of sialic acid residues and decreased biological activity of FSH by 97 percent. The conclusion of early workers that the active portion of FSH is in the carbohydrate moiety was reaffirmed by Cahill, Shetlar and Payne (1962). Incubation of FSH with alpha-amylase resulted in loss of biological activity and a decrease in bound hexose. Also, beta-glucuronidase decreased the concentration of bound uronic acid and destroyed biological activity of FSH.

## Carriers for Follicle Stimulating Hormone

### Physiological Saline

Several solutions and their combinations, water (Fevald et al., 1931), physiological saline (McShan and Meyer, 1937, 1955; Steelman and Pohley, 1953), 0.9 percent benzyl alcohol (Segaloff, 1962) and saline plus 0.5 percent gelatin (Steelman et al., 1959), have been used as carriers for FSH administered to animals. However, physiological saline (PSS) has persisted as the carrier for this hormone (FSH-P, Armour-Baldwin Company, 1969).

### Augmentation by Carrier Additives

The possibility of enhancing the activity of gonadotropic substances through utilization of various carriers or carrier-additives has been investigated by several researchers. McShan and Meyer (1937) tested whole blood, hemolyzed erythrocytes, hemoglobin and heme mixed with anterior pituitary extracts. Evidence of augmentation by each of these when mixed prior to injection was indicated by greatly increased ovarian weights in immature female rats. However, Casida et al. (1943) found the addition of heme to unfractionated pituitary extracts failed to increase quantitative responses of ovaries in calves or cows.

Aluminum, iron and zinc hydroxides increased the effectiveness of sheep pituitary gonadotropin in rats (McShan and Meyer, 1945). They suggested that adsorption of the hormone proteins by the hydroxides slowed the release of the active hormone from the injection site which

resulted in a continuous stimulation of the ovaries. McShan and Meyer (1953) dissolved sheep pituitary extracts in a one-half saturated NaCl solution and obtained greater follicle stimulation than when the same extracts were dissolved in PSS. It was concluded that augmentation by concentrated NaCl and sucrose solutions was due to a slowed release of the hormones from the injection site. Implants of sheep pituitary preparations combined with wax were used by Umbaugh (1949) for administration to cattle. An intravenous injection of the same extract 4 days following the wax implant was shown necessary to achieve superovulation.

## Carboxymethylcellulose

Carboxymethylcellulose (CMC) has been suggested as a carrier for FSH (Vincent, 1970). The number of corpora lutea (CL) observed following single injections of gonadotropins in cattle were less variable with CMC as the carrier than PSS (Mills and Vincent, 1969). On the other hand, CMC at a ratio of 2 mg FSH:1 ml CMC showed no apparent advantage over FSH in saline (Mills et al., 1971). Five mg of FSH in 5 ml CMC were injected once per day on days 10 and 11 of an 11-day synchronization treatment with medroxyprogesterone acetate (MAP) and 5 mg FSH in 5 ml water were injected twice daily on days 10 and 11. A significantly greater number of corpora lutea and a slightly greater number of live embryos were observed at slaughter from FSH in CMC than from twice as much FSH in water (Reynolds et al., 1970). Ratios of FSH to CMC other than 1 mg:1 ml were not reported.

Commercially, the term carboxymethylcellulose is applied to an anionic water-soluble cellulose ether which is the sodium salt of carboxymethylcellulose. It is a physiologically inert colloid. Cellulose gum, CMC or sodium cellulose glycolate are other terms used to describe the same compound (Savage, Young and Maasberg, 1954; Hercules Inc., 1971). Commercial CMC is available in high, medium or low viscosity types with degrees of substitution ranging from 0.38 to 1.40 (Hercules Inc., 1971). It is generally considered to contain about 0.5 carboxymethyl groups per C6-unit (Noller, 1965). McBurney (1954) reported growth of microorganisms in water soluble CMC produced a variety of undesirable properties. Heat sterilization and the addition of bacteriostats were cited as effective methods for control of this type of degradation.

Sober and Peterson (1958) and Harper (1965) described CMC as a cation-exchange material derived from cellulose which has been extremely successful in the purification of proteins by selective adsorption and elution. Peterson and Sober (1956) suggested that the large size of proteins prevents penetration into adsorbents so an enormous surface provided by very fine particles is necessary for high adsorption capacity. CMC was tested with horse carbon monoxide hemoglobin in 0.01 M sodium phosphate at pH 6.0 and was shown to adsorb quantities approximately equal to its own weight under these conditions. Separation of proteins was suggested a result of different isoelectric points together with different secondary adsorption forces on the "surface" of the resin particles (Ketchner,

1957). He stated further that much of this work has been carried out with carboxylic acid resins based on the theory that weak acid resins are less likely to cause denaturation of proteins than strong acid resins since the adsorption affinity can be moderated by changing the pH of the medium. However, Raacke and Li (1957) and McShan and Meyer (1955) used synthetic ion exchangers successfully as aids in the purification of FSH without apparent loss of biological activity.

Highly purified preparations of FSH have been obtained with columns of DEAE cellulose and CMC (Steelman et al., 1956, 1959; Ellis, 1958; Steelman and Segaloff, 1959; Ward et al., 1959; Cahill et al., 1961). Under the conditions described, DEAE cellulose retained the FSH fraction most strongly and CMC showed a stronger affinity for LH than FSH.

## Procedures for Bio-Assay of FSH Activity

Rats and mice have been the most acceptable animals for quantitative evaluation of the follicle stimulating activity of gonadotropic preparations. Several anatomical and behavioral changes in laboratory animals have been used as end-points in the estimation of physiological activity of gonadotropins.

Engle (1931) observed an abnormally early appearance of the vaginal orifice followed by induced estrus in immature mice implanted with anterior pituitaries. Smith (1926), Engle (1927) and Smith and Engle (1927) used pituitary transplants to demonstrate marked ovarian weight increases and superovulation in adult females as well as precocious sexual maturity in immature animals. Also, Smith and

Engle (1927) suggested the observed increase in uterine weight was a secondary effect as treatment of ovariectomized females resulted in no effect on uterine weight or the opening of the vagina. Increases in uterine weight, follicle size and ovarian weight in immature, intact or hypophysectomized rats or mice were recommended as physiological end-points for assay of FSH activity (Zarrow, Yochim and McCarthy, 1964).

Hypophysectomized animals which allow disregarding the effects of the test animals' own pituitary glands are preferable to intact immature animals (Chow et al., 1939; Greep et al., 1940). However, Steelman and Pohley (1953) and Zarrow et al. (1964) recommended mixing human chorionic gonadotropin (HCG) with FSH to negate the endogenous LH, and LH impurities, which may be in the FSH by saturating the animal with LH-like activity. Simpson, Li and Evans (1951) had previously demonstrated a synergistic action of LH and FSH.

Slight variations in injection procedures have been used by different workers. Fevald et al. (1933), McShan and Meyer (1938, 1940) and Leonora et al. (1956) began injections of 0.5 ml volumetric dosages on the evening of the first day followed by twice daily injections the next 4 days with sacrifice and removal of desired organs the morning of the sixth day. However, the procedures of Steelman and Pohley (1953) have persisted as the most widely recommended for bio-assay of FSH activity (Segaloff, 1962). The procedures of Steelman and Pohley (1953) are summarized as follows: HCG was used as an augmentation factor to reduce the influence of endogenous LH as well as LH

impurities in the FSH. The recommended procedure for administration of FSH preparations to immature (21 or 22-day old) intact, female rats was to divide the total dose desired into 0.5 ml subcutaneous injections three times per day for 3 days with sacrifice and removal of ovaries at 72 hours after the initial injection. However, in some of their evaluations, preparations were injected once per day for 3 days with sacrifice at 72 hours. Five or six animals were lotted to each treatment group and the combined weight of both ovaries was considered the parameter of response since differences between left and right ovaries were shown nonsignificant. It was noted that animals of the same ages showed no significant differences in ovarian responses despite variations in body weight. The dose-response curves were shown to be linear within a narrow total dose range of 0.05 to 0.30 mg Armour Standard. At levels above 0.30 mg, the curves began to plateau.

## Induced Multiple Ovulation in Beef Cattle

### Natural Phenomena

It is generally agreed that the incidence of multiple births in cattle is low. Johansson (1932) reported from observations of nearly a million births the incidence of natural twinning in beef breeds was 0.44 percent and in dairy breeds only slightly higher at 1.88 percent. Later, Richter (1955) reported 3.2 percent of 59,000 calvings were multiple births.

Following the discovery that pituitary transplants would stimulate ovarian follicular growth in laboratory animals (Smith, 1926; Engle, 1927, 1931; Smith and Engle, 1927), exogenous gonadotropins were used to produce multiple ovulations in cattle (Casida et al., 1943; Hammond and Bhattacharya, 1944). Subsequent to these initial efforts, research related to superovulation in cattle has been bi-directional. Some workers (Willett, McShan and Meyer, 1948, 1952; Rowson, 1951; Scanlon, 1971; Rowson, Lawson and Moor, 1971) have attempted to achieve the greatest number of fertilizable ova possible at a single estrus for laboratory study or for ova-transplant. Conversely, the efforts of other researchers (Schilling and Holm, 1963; Bellows, Anderson and Short, 1969; Laster et al., 1971) have been directed toward achieving limited ovarian stimulation resulting in a theoretical optimum of two calves at parturition. One of the major problems in the latter approach has been the variation in response of ovulation rate in similar cattle treated alike (Hammond and Bhattacharya, 1944; Dziuk et al., 1958; Hafez, Jainudeen and Lindsay, 1965; Mills and Vincent, 1969).

The general observation, that ovulatory responses to exogenous gonadotropin treatments are highly variable and unpredictable, has suggested a need for further study of the natural physiological and endocrine phenomena related to ovulation. A broader understanding of sequential physiological changes and coincident hormonal secretions and balances could lead to more sound bases from which to develop practical treatments with exogenous compounds.

Rajakoski (1960) described the histological changes of the ovary during the normal estrous cycle in cattle. It was concluded that growth of follicles >5 mm occurred in two waves during a single estrous cycle. The first follicle began growth between the first and fourth days of the cycle and the second between days 12 and 14. Both growth waves resulted in single follicles of pre-ovulatory size. However, the follicles of the first wave became atretic beginning about day 12 and the follicle of the second wave ovulated at the succeeding estrus. Marion, Gier and Choudary (1968) reported that follicular growth from 0.5 to 1.0 mm in cattle was rapid, but development from 1.0 to 12.0 mm appeared continuous and without periods of acceleration until 18 to 24 hours prior to ovulation when rapid expansion of the follicle occurred.

In an extensive literature review, Hisaw (1947) offered tentative conclusions concerning ovulation in the bovine: (1) Pre-ovulatory enlargement and rupture of ovarian follicles is by joint action of FSH and LH, (2) Neither FSH or LH alone cause ovulation, (3) Ovulation is initiated by increased secretion of pituitary gonadotropins, primarily LH, (4) Increased pituitary hormone is caused by the activity of estrogen and probably progesterone, and (5) The sensitizing effect of FSH may assist the ovulatory reaction. Rakha and Robertson (1965) concluded from studies of pituitary FSH and LH that both play a role in the induction of ovulation in cattle. A significant decrease in pituitary FSH (27 percent) and LH (61 percent) was shown to occur during the period 0 to 18 hours after the onset of

estrus. These decreases suggested the amounts of FSH and LH secreted may vary and also their ratio to each other may be important. A slightly different time of FSH release was observed by Robertson and Rakha (1966) who reported a steady decrease in pituitary FSH beginning 8 hours prior to and continuing to 6 hours after the onset of estrus. No change was observed in the LH content of the pituitary during the 12 hours preceding the onset of estrus. However, LH discharge started at the onset of estrus and was complete in 6 hours. It was concluded that the release of gonadotropins leading to ovulation occur during the first few hours after the onset of estrus. Henricks, Dickey and Niswender (1970) reported very similar observations for LH, but Sprague et al. (1971) reported LH peaks coincident with the onset of estrus. Gordon, Williams and Edwards (1962) had hypothesized earlier that where single or multiple ovulations occurred spontaneously in the cow it seemed likely the result of a slow and continuous rather than a momentary and explosive discharge of FSH.

Progesterone in the corpus luteum (CL) and ovarian venous blood decreased rapidly during proestrus from a maximum at days 14 to 16 of the bovine estrous cycle (Gomes and Erb, 1965). In agreement, Stabenfeldt, Ewing and McDonald (1969) reported plasma progesterone levels in 21-day cycle cows increased rapidly days 3 to 8 with a much slower rate of increase days 8 to 17 followed by a rapid decrease (50 percent less than the previous day) on days 18, 19 and 21 varying with individual cows. A similar pattern was observed in swine

(Stabenfeldt et al., 1969) with the suggestion that high levels of circulating progesterone may suppress follicular growth so approximately 1 week is required between CL regression and ovulation.

Relationships between ovarian steroids and pituitary gonadotropins were studied by Gay and Bogdanove (1969). Their work with rats suggested estrogen and progesterone impaired both the synthesis and release of FSH as estradiol benzoate and progesterone injections consistently lowered plasma FSH titers without altering FSH stores in the pituitary. This indicated an impairment of both synthesis and release of FSH. Labhsetwar (1969) reported that progesterone in the rat tended to decrease both the release and pituitary synthesis of LH and suggested the same may be true for FSH. However, it was suggested previously (Hansel and Trimberger, 1952) that progesterone played a role in LH release because subcutaneous injections of 5 to 10 mg of progesterone at the beginning of estrus hastened the ovulatory process in dairy heifers. Recent studies of serum LH levels in cows (Howland et al., 1971) demonstrated that a release of LH can be induced by estrogen and this release is not affected by progesterone treatment. Estrus was first detected and serum LH was elevated in all cows between 16 and 24 hours post-injection of 10 mg estradiol 17-beta.

## Hormone-Time-Dose-Animal Relationships

The complexity of hormonal induction of multiple ovulation in cattle was demonstrated by Lamond (1970) where plane of nutrition, fasting, progesterone treatment, season, dose and time of

administering PMS in relation to progesterone treatment were all reported to influence follicular development and ovulation.

Hormones. In attempts to obtain ovarian stimulation, gonadotropic activity from the following sources have been utilized: Whole pituitary transplants (Smith, 1926; Engle, 1927, 1931; Smith and Engle, 1927); pituitary extracts (Pincus, 1940; Casida et al., 1943; Hammond and Bhattacharya, 1944; Umbaugh, 1949) and whole and purified pregnant mare serum (Cole and Hart, 1930; Goss and Cole, 1931; Pincus, 1940; Hammond and Bhattacharya, 1944). There were significant differences in ovarian responses between different "batches" of pituitary extract injected to ewes (Murphree et al., 1944) and PMS injected to cattle (Hafez et al., 1964). A greater number of follicles were developed in cattle from whole PMS than from commercially processed PMS (Rowson, 1951). If HCG was injected intravenously subsequent to PMS, there was an increase in ovulation rate of developed follicles. It has also been suggested that unfractionated ovine FSH was superior to HCG in causing ovulation in cattle (Willett et al., 1948). Avery, Fahning and Graham (1962) used swine FSH plus a single injection of LH to induce superovulation in immature, mature and also pregnant cows. No further increase in ovulation rate was shown by the addition of prolactin or a second injection of LH. On the other hand, it has been reported that exogenous LH is not required to cause ovulation of developed follicles in the cow (Dowling, 1949; Hafez et al., 1965). This is apparently due to relatively high levels of

LH in bovine serum on day of estrus (Niswender et al., 1969; Sprague et al., 1971).

Dziuk et al. (1958) concluded that more consistent follicular growth stimulation was observed from injections of swine FSH than PMS, but some follicles failed to ovulate. LH injections following swine FSH did not improve ovulation; however, mixing of FSH and LH at injection gave more desirable results.

Investigations concerning the effects of preparation and source of gonadotropins are apparently decreasing. The gonadotropins used by recent workers in the area of multiple ovulation in beef cattle have been largely limited to FSH (Bellows et al., 1969; Mills and Vincent, 1969; Vincent and Mills, 1969; Reynolds et al., 1970; Mills et al., 1971) and PMS followed by HCG (Schilling and Holm, 1963; Laster et al., 1971; Turman et al., 1971).

Dose. A great deal of work has been conducted in an attempt to determine optimal dosages of exogenous hormones. The relationship of total gonadotropin to number of administrations is apparently important. Minimal amounts of PMS which would produce sexual maturity in rats in single doses were insufficient to do so when distributed over a 4-day period (Cole, Guilbert and Goss, 1932). On the other hand, Parlow and Reichert (1963) reported twice daily injections were clearly more effective than single injections in rat bio-assays of porcine FSH and to a lesser degree in the case of ovine, human, rat and equine FSH. In agreement, twice daily injections of FSH to cattle

resulted in higher ovulation rates and more cows with multiple CL than once daily injections at higher doses (Vincent and Mills, 1969).

Gordon et al. (1962) reported increased multiple ovulation with increased doses of PMS on day 16 or 17 of the estrous cycle in cattle, but that as dosage increased variability of ovarian response also increased. Willett et al. (1948) used 20, 30 and 40 g equivalents of ovine FSH in single subcutaneous injections each day for 5 days and intravenously on day 6. Higher doses responded in greater ovarian stimulation.

Lamond and Hill (1969) injected varying amounts of PMS (130 to 3,500 IU) into heifers on day 14 of an 18-day synchronization treatment with melengesterol acetate. It was concluded that the effective dose range of PMS for controlling the number of ovulations to approximately two per estrus may be very narrow. The most successful attempts to achieve limited multiple ovulations in cattle with PMS injections have been from 1,500 IU administered early (days 3 to 6) in the estrous cycle followed by 2,000 IU later (days 16 to 18) in the cycle (Schilling and Holm, 1963; Laster et al., 1971; Turman et al., 1971).

Extensive superovulation was produced in both cows and calves by Avery et al. (1962) for ova-transplant studies. Twenty mg FSH and 5 mg LH were injected subcutaneously on day 1 following synchronization treatment followed by 10 mg FSH and 5 mg LH on days 2, 3 and 4 with 100 mg of LH injected intravenously on day 5. In animals which were not synchronized, the same sequence of treatments was initiated on

day 16 of the normal estrous cycle. A significant linear dose-response relationship was shown in cattle between dosage increase and ovarian stimulation (Bellows et al., 1969). Their extensive study of responses to various dosages of FSH was conducted in heifers which were synchronized for 11 days with medroxyprogesterone acetate (MAP). Estradiol valerate was injected on day 2 followed by twice daily injections of FSH on days 8, 9, 10, 11 and 12 of the synchronization treatment. Treatments and ovulatory responses as shown in the following table indicated overstimulation at 12.5 mg or more. The total dose of 6.25 mg appeared optimum.

| | Total FSH dosage (mg) | | | |
|---|---|---|---|---|
| | 3.12 | 6.25 | 12.5 | 25.0 |
| No. of animals | 8 | 8 | 8 | 7 |
| Ovulations | 9 | 17 | 64 | 102 |
| Average | 1.2 | 2.1 | 8.0 | 14.6 |
| Range | 1-2 | 1-4 | 3-14 | 1-32 |

No evidence was noted indicating a relationship between size of heifer and response to FSH treatment.

Mills and Vincent (1969) reported 10 mg FSH in 3.6 or 5.0 ml CMC resulted in an average of 2.0 and 2.3 CL, respectively, but 10 mg FSH in 2 ml saline produced an average of 3.3 CL with highly variable individual responses in cattle. Eight mg FSH in 4 ml CMC resulted in more melengesterol acetate (MGA) synchronized heifers in heat within 2 to 6 days and more multiple CL than 6 mg FSH in 3 ml saline or CMC or 10 mg FSH in 5 ml CMC (Mills et al., 1971). However, degree of stimulation and uniformity of response were both poor. FSH injections

of 6.25 mg administered twice daily to beef cows synchronized with norethandrolone injections resulted in an average of 2.2 ovulations, but only 50 percent of the cows showed 2 or 3 ovulations and pregnancy rate was only 50 percent (Vincent and Mills, 1969). Reynolds et al. (1970) reported 5 mg FSH in 5 ml CMC administered once per day on days 10 and 11 of an 11-day MAP synchronization treatment resulted in over-stimulation with an average of 8.6 CL; but 1 mg FSH in 1 ml water administered twice daily, days 8 through 12, resulted in a mean observed CL of 3.4. No stimulation was obtained by twice daily injections of 5 mg FSH in 5 ml water on days 10 and 11.

Time. Hammond, Jr., Hammond and Parkes (1942) observed PMS injections to anestrous sheep in the absence of a CL resulted in ovulation without expressed estrous behavior. If an active CL was present, both ovulation and heat were suppressed. They reported that horse pituitary extract and PMS injected in the presence of a regressing CL resulted in psychic heat. A recommendation was made that horse pituitary extract should be injected approximately 3 days before the CL is expected to regress. Good conception in sheep was reported by Wallace (1954) if PMS was injected 5 days or less prior to onset of estrus. However, poor conception was observed if PMS was administered earlier in the cycle. This suggested that optimum fertility was obtained when PMS was injected 2 or 3 days prior to estrus and it was recommended that injections should be administered on day 14 of the cycle. Murphree et al. (1944) reported that gonadotropin stimulation in 24 "follicular" ewes produced an average of 24 CL with 14.6 eggs

recovered per ewe. Seven of the 24 produced only nonfertilized eggs, but an average of 9 fertilized eggs were recovered from the other 17. Five ewes treated during the "luteal" phase of the estrous cycle produced an average of 12.4 CL with 5 eggs recovered per ewe and 9 anestrous ewes produced 9.3 CL, each with less than 3 eggs recovered per ewe. None of the eggs recovered from the luteal phase or anestrous ewes had been fertilized. Similar results, showing a marked difference in fertility, were reported to be associated with day of treatment in swine (Tanabe et al., 1949).

Marden (1952) reported, from work with calves, that pituitary extracts plus HCG or LH resulted in excessive follicular growth without ovulation. The presence of a CL was cited as being of prime importance in completing ovulation of stimulated follicles, since superovulation was obtained from a second series of injections after an interval of 15 days. PMS injections on days 6 and 16 to 18 with enucleation of the CL at last injection resulted in 70 percent of treated cows responding with double or triple ovulations (Schilling and Holm, 1963). Willett et al. (1948) reported greater ovarian stimulation in heifers by ovine FSH during the follicular phase (day 16) than the luteal phase (day 4). In the presence of an active CL, injection with gonadotropins plus insemination resulted in pyometria and no fertilization. Five heifers treated during the luteal phase produced 34 nonfertilized eggs, but 5 treated during the follicular phase produced 43 eggs, 74 percent of which were cleaved (Willett, McShan and Meyer, 1952). Dowling (1949) found injections of gonadotropin in cattle 4 to 5 days prior to

the onset of estrus resulted in less variable responses than injections 7 to 9 days prior to the onset of estrus.

Dosages of 1,500 to 5,000 IU PMS at various intervals from 5 days prior to 3 days post CL expression resulted in up to 30 ovulations per cow, but rupture of follicles during CL expression was cited as problematic (Hammond and Bhattacharya, 1944). Injections of 1,500 IU PMS toward the end of the cycle produced different responses than the same dose combined with CL removal. This dosage administered 3 to 4 days before heat resulted in mostly double ovulations. Avery et al. (1962) used a series of FSH and LH injections beginning day 16 of natural cycles or on day 1 following progesterone treatment to obtain superovulation in immature, mature or pregnant cattle. On the other hand, Hafez et al. (1965) reported injections of PMS during the luteal or follicular phases of the cycle did not influence treatment estrous cycle lengths or ovulation responses, but there was no apparent advantage in initiating treatment earlier than the sixteenth day of the cycle or from expression of the CL.

Turman et al. (1971) reported relatively good controlled ovulation rates and conception in cattle where PMS was injected on days 3, 4, 5 or 6 with another injection on days 16, 17 or 18 of the normal cycle plus an intravenous injection of HCG on day of estrus. It was later concluded that PMS on days 5 and 17 of the cycle followed 3 days later by HCG resulted in the most controlled and repeatable ovulation rates (Laster et al., 1971).

Following the theory that ovulations occur as a result of a slow, continuous discharge of FSH (Gordon et al., 1962), Bellows et al. (1969) used twice daily injections of FSH on days 8, 9, 10, 11 and 12 of an 11-day estrus synchronization treatment procedure to produce a highly desirable average of 2.1 ovulations per heifer.

The general conclusion that exogenous gonadotropins should be administered during the follicular phase of the cycle appears feasible in view of the observation by Rajakoski (1960) that follicular growth occurs in two waves, the last of which ovulates.

Animal. The early observations in cattle (Hammond and Bhattacharya, 1944) and sheep (Murphree et al., 1944) of considerable variation in ovarian responses between animals treated alike were reaffirmed for cattle in an extensive literature review and study by Dziuk et al. (1958). A relationship of response to individuality of animals was demonstrated in cattle by Asdell, DeAlba and Roberts (1945), as animals in which it was easy to detect heat prior to ovariectomy required lower levels of stilbestrol to induce heat after ovariectomy. Variability in ovarian response between cattle treated alike persists (Hafez et al., 1965; Mills and Vincent, 1969).

Genetic interaction may be an important consideration in future studies of ovarian stimulation. Rollins and Cole (1952) compared the variability of inbred and random bred rats used in gonadotropin assays. It was demonstrated that 80 to 85 percent of the variance in random bred rats was genetic in origin and they suggested five to seven times as many random bred rats were required to provide a given level of

precision in bio-assays of gonadotropins. Mice differing at a single genetic locus released different numbers of ova in response to exogenous gonadotropin stimulation (Wolfe, 1971). Also, Zarrow, Christenson and Eleftheriou (1971) reported a significant strain difference in mice both in ovarian response to PMS and to a combination of PMS and exposure to adult males. Edwards and Fowler (1960) found some mice capable of ovulation or superovulation without HCG following PMS, but HCG was required to produce ovulation in other animals.

An important breed difference was shown in ovulation rate in swine (Kirkpatrick et al., 1967). However, no follicle stimulating treatment was included and, therefore, results were not reported relative to breed x gonadotropin relationships. Land (1970) reported a significant positive correlation between length of estrus at time of mating and litter size in sheep. Wallace (1954) reported from work with sheep that breed or strain differences may be important in the extent of ovulatory response to PMS. In a similar study, if a given level of fertility was associated with certain breeds, sheep with an apparently natural lower fertility level showed a greater response to given doses of PMS than those breeds considered more highly fertile (Gordon, 1958).

## Refractoriness

A complete description of the interrelationships of animals, compounds and treatment regimes to the development of refractoriness to exogenous gonadotropins has not been presented to date. However,

the frequency of its incidence suggests the problem of refractoriness is worthy of consideration in studies related to superovulation in cattle.

Contrary to reports by a majority of workers that animals become refractory to repeated injections of gonadotropins, Umbaugh (1949) reported that superovulation may be induced repeatedly in the same cow. In agreement, Dziuk et al. (1958) found no consistent reduction in response by cows repeatedly superovulated with PMS but did report two cases of anaphylaxis. Relative maturity of animals at initial treatment may be a factor in the extent of refractoriness developed. Marden (1952) reported superovulation with the formation of multiple CL was obtained in calves by repeating FSH injections after a 15 day interval. The first treatment resulted in excess follicular growth without ovulation.

Several different gonadotropins can result in reduction in response to repeated stimulation. A highly significant decline in number of CL was observed in cattle with successive superovulations by five different compounds (Willett and Buckner, 1953). Increased dosages were shown to partially, but temporarily, overcome refractoriness. In agreement, Jainudeen et al. (1966) reported that the same doses repeated at subsequent estrous cycles in cattle failed to stimulate ovaries. However, an increased dose of PMS after a delay of 5 to 7 months resulted in ovarian responses similar to those of the initial treatment. It was also noted that the antigonadotropic activity developed in PMS treated cows inhibited the follicle

stimulating properties of PMS but showed no adverse effect on endogenous gonadotropins. Repeated superovulation with FSH and PMS resulted in a decline in follicular and ovulatory response in calves as well as mature cows (Onuma et al., 1969). Contrary to reports by Hafez et al. (1964) and Jainudeen et al. (1966) of apparent complete refractoriness, Laster et al. (1971) reported only partial refractoriness to a second sequence of PMS treatments with 51 percent of the heifers and 37 percent of the cows multiple ovulating.

Mice given one superovulation treatment can respond to a second shortly thereafter, but the number of eggs is decreased and varies greatly between mice (Edwards and Fowler, 1960). Some strains of mice develop a refractoriness more rapidly than others (Lin and Bailey, 1965). Mating was shown to result in ovulation in hormone primed mice which were refractory after 10 successive superovulations which suggests normal responses to endogenous gonadotropins. Refractory reactions develop rapidly in ewes but appear to decrease gradually with time (Hulet and Foote, 1969). After one year, responses to PMS were intermediate between PMS refractory ewes and ewes receiving their first PMS injection.

Level as well as purity and type of gonadotropin used as sequential stimulation may be an important consideration in the development of refractoriness. A first cycle injection of 2,000 IU PMS followed by 3,000 IU at the second cycle resulted in fewer ovulations in cattle than the reverse dose sequence (Scanlon, 1971). An apparent inverse reaction to increased dosages of PMS was indicated

by the appearance of antigonadotropin activity in sheep (Pigon, Clegg and Cole, 1960). Antigonadotropin activity was detected the second week with twice weekly injections of 200 IU of PMS but not until the third and fifth weeks when doses were increased to 500 and 1,000 IU, respectively.

Woods and Simpson (1961) reported as purity of FSH increased antagonism to increased follicular growth decreased in rats. Results of two bio-assays for antihormones suggested that decreased ovulation responses in rabbits were due to hormonal refractoriness which was more pronounced in animals treated with PMS and HCG than FSH and LH (Maurer, Hunt and Foote, 1968).

## Synchronization

The apparent importance of restricting ovarian stimulatory treatments to definite stages of the estrous cycle leads to consideration of estrus synchronization as an aid to management. Successful synchronization of estrus in cattle has been obtained by the use of progesterone (Christian and Casida, 1948; Lamond, 1964; Shelton and Moore, 1966), 6-chloro delta 6-17 acetoxyprogesterone (Wagner et al., 1968), norethandrolone (Mills and Vincent, 1969; Crofton, Warnick and Bazer, 1971), dehydroxyprogesterone acetophenide (Wiltbank et al., 1967; Wiltbank and Kasson, 1968), 6 alpha-methyl-17 alpha-acetoxyprogesterone (Bellows et al., 1969; Reynolds et al., 1969, 1970) and melengesterol acetate (Zimbelman and Smith, 1966; Reynolds et al., 1969; Boyd, 1970; Hill et al., 1971). Estrogen injections were shown luteolytic (Wiltbank, 1966) and hence can decrease the length of time

progestational compounds are administered for estrus synchronization.

Fertility at first estrus following synchronization has been highly variable but generally lower than nonsynchronized controls. Some of the reasons for suppressed fertility may be a residual effect of the progestin which causes responses similar to the presence of a CL (Hammond et al., 1942; Hammond and Bhattacharya, 1944; Rowson, 1951), altered rate of ova transport through the oviduct (Rowson, 1951; Lamond, 1964) and altered sperm transport (Cullen, Hovell and Shearer, 1968). Fertility at the second estrus post-synchrony is apparently normal (Trimberger and Hansel, 1955).

## Other Aspects of Multiple Ovulation

Several aspects of the reproductive processes involved in the production of twin calves by hormonal treatment will need to be investigated further before this practice becomes commercially feasible.

A tendency toward premature birth has been observed in cattle carrying twins (Hammond, Jr., 1949; Turman et al., 1971). It has been suggested that the uterine horn of the bovine is rarely capable of maintaining more than two fetuses and that palpation at 6 weeks can result in a severe loss (Gordon et al., 1962). In agreement, Rowson et al. (1971) demonstrated a higher embryonic death rate when two eggs were transplanted to a single uterine horn rather than one egg per horn. On the other hand, Kodituwakku and Hafez (1970) suggested that

high prenatal mortality in cattle following PMS is caused by hormonal imbalance rather than overcrowding in utero.

The length and expression of estrus may be a problem following superovulation as Dziuk et al. (1958) found that, in most cases, cattle treated with gonadotropins did not exhibit normal estrous behavior. Turman et al. (1971) reported a high frequency of retained placentas in cows having multiple births, but this did not appear to be associated with rebreeding efficiency. However, 25 percent of the cows nursing twins did not rebreed while lactating, but conception did occur within 6 weeks following weaning.

Nutritive status of the stimulated animal has an apparent important influence on responses to gonadotropins. In an evaluation of bio-assay precision in rats, Rollins and Cole (1952) reported variations in feed and water intake during the period between injection and autopsy contributed significantly to variability in ovarian weights. The addition of corn oil or glucose to a basal ration for swine produced an increase in ovulation rate (Kirkpatrick et al., 1967). An increase in PMS induced ovulations in mice expressing compensatory growth following a period of limited nutrition was observed by Lamond and Bindon (1969). Adult mice fed ad libitum following restricted feeding showed higher fecundity than those returned directly to a normal ration following restriction or by others where feed was increased slowly. Wallace (1954) cited nutritive status of ewes as an important factor affecting the extent of ovulation rate in response to PMS treatments. Heifers on a high

plane of nutrition produced more ovulations in response to PMS treatments than did those on a low plane of nutrition (Lamond, 1970).

# SECTION I

## EVALUATION OF CMC AS A CARRIER FOR FSH INJECTIONS

## Introduction

A need for a diluent which would prolong absorption of gonadotropin injections when attempting to control induced multiple ovulations in the cow has been suggested (Vincent, 1970). In apparent support of this suggestion, Bogdanove and Gay (1969) reported the biological half-life of exogenous follicle stimulating hormone (FSH) in hypophysectomized rats was relatively short (48 to 93 minutes). Also, Gordon, Williams and Edwards (1962) reported that, when single or multiple ovulations occur spontaneously in the cow, it seems likely they result from a discharge of FSH which is slow and continuous rather than momentary and explosive.

Experiments with whole blood, hemolyzed erythrocytes, hemoglobin and heme (McShan and Meyer, 1937); aluminum, iron and zinc hydroxides (McShan and Meyer, 1945); concentrated NaCl (McShan and Meyer, 1953) and wax (Umbaugh, 1949) as carriers for gonadotropins resulted in an enhancement of follicle stimulating activity. However, physiological saline (PSS) remains the standard vehicle for FSH preparations (FSH-P, Armour-Baldwin Company, 1969).

Recently, carboxymethylcellulose (CMC) has been utilized as a carrier for FSH in attempts to achieve controlled superovulation in beef cattle (Reynolds et al., 1970; Mills and Vincent, 1969; Mills et al., 1971). The gonadotropic effects of different ratios of hormone to carrier were not compared, but Mills and Vincent (1969) reported variability in number of corpora lutea (CL) following single injections

of gonadotropins was reduced with CMC as the carrier when compared to PSS.

CMC is an anionic water-soluble polymer, a physiologically inert colloid (Hercules Inc., 1971). It has been used as a cation exchanger in the purification of proteins by selective adsorption and elution (Sober and Peterson, 1958; Harper, 1965). It has also been used successfully in sequence with diethylaminoethylcellulose (DEAE cellulose) in the preparation of highly purified FSH (Steelman et al., 1956; Ellis, 1958; Ward, McGregor and Griffin, 1959; Cahill, Payne and Sheltar, 1961).

The purposes of this study were to compare CMC and PSS as carriers for FSH and to evaluate quantitative relationships of FSH and CMC.

## Materials and Methods

### General

Carboxymethylcellulose (CMC)[1] was dissolved in distilled water to develop a 1 percent solution. Dissolution was by low-shear agitation with periodic light stirring for approximately 8 hours. The solution was then sterilized by autoclaving at 115 to 120 C and 10 to 15 pounds pressure for 20 minutes to avoid microbial degradation (McBurney, 1954; Hercules Inc., 1971) and to provide a material suitable for animal injections.

Follicle stimulating hormone (FSH-P)[2] was activated by the addition of 2 to 5 ml of sterile, cold, 1 percent CMC solution or physiological saline (PSS) to the lyophilized powder. Desired quantities were then extracted for further dilution to meet selected ratios of FSH to carrier. Materials were kept cold (7 C) during preparation and final solutions of desired ratios were labeled and refrigerated for 1 to 2 hours to allow possible ionic binding of FSH and CMC.

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[1]CMC (sodium carboxymethylcellulose), Hercules Powder Company, Wilmington, Delaware. Hercules CT grade, 75 percent sodium carboxymethylcellulose, balance salt of reaction, Lot #7838.

[2]FSH-P (Follicle stimulating hormone-pituitary), Armour-Baldwin Laboratories, Omaha, Nebraska. Supplied as a lyophilized powder obtained from pituitary glands of domestic animals. Each 10 cc vial is listed to contain the equivalent of 50 mg Armour Standard FSH units and levels reported are based on this value.

Immature Sprague-Dawley[3] female rats were purchased and lotted to treatment groups randomly at 19 days of age, placed in small cages of three each and fed Purina Lab Chow ad libitum until sacrificed. Three days were allowed for adjustment to local environmental influences. Thus, in accordance with procedures recommended by Steelman and Pohley (1953), the experimental animals were 22 days old when initial subcutaneous injections were administered.

Bio-assay procedures of Steelman and Pohley (1953) were modified as follows:

1. In no case was a material of unknown FSH activity used so a standard curve for comparison of known to unknown activity was not required.
2. Their dose-response curves were shown to be linear within a narrow range of 0.05 to 0.30 mg with a tendency to plateau at higher levels. In the present study, maximal responses with minimal hypertrophy were desired. Therefore, 0.50 mg of FSH was selected as the individual dose for all FSH treatments.
3. Instead of thrice daily injections of small aliquots for 3 days, a single injection of the desired dose was used.
4. The effects of carrier and hormone-carrier ratios on the activity of commercial FSH were of major concern. Hence, the HCG augmentation procedure was omitted.

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[3]Sprague-Dawley Company, Madison, Wisconsin.

5. In order to study time-carrier relationships to parameters of response, animals were sacrificed at 0, 24, 48, 72 and 96 hours post-injection instead of at 72 hours after the first injection.

In accordance with procedures of Steelman and Pohley (1953), six animals from each treatment group were sacrificed at each of the four time intervals. Only six animals were measured to serve as 0-hour controls for all treatments in each experiment.

Two parameters of response to treatments were utilized: (1) The combined weight of trimmed ovaries and (2) The trimmed uterus. These tissues, weighed to the nearest 0.1 mg, were considered measures of primary and secondary responses, respectively (Steelman and Pohley, 1953; Zarrow, Yochim and McCarthy, 1954).

The animals were sacrificed by ether asphyxia. The ovaries and uterus were dissected from the body and placed in a petri dish with physiological saline to reduce cellular weight changes during trimming. Remnants of the broad ligament, the oviducts and adherent fatty tissues were removed with the aid of a dissecting microscope. The oviducts were discarded and not weighed with either the ovarian or uterine tissues.

A series of four bio-assay experiments were conducted during late 1970 and early 1971 with treatments administered as outlined in table 1. Treatments which include FSH are expressed as mg FSH:ml carrier.

TABLE 1. EXPERIMENTAL DESIGN SHOWING TREATMENTS AND DOSAGES FOR BIO-ASSAY, EXPERIMENTS I THROUGH IV

| Experiment | Treatment[a] (mg FSH:ml carrier) | Mg FSH | Ml PSS | Ml CMC | Dosage[b] (ml) |
|---|---|---|---|---|---|
| I | 0:1 | -- | 1.0 | -- | 0.167 |
| | 0:1 | -- | -- | 1.0 | 0.167 |
| | 3:1 | 3.0 | 1.0 | -- | 0.167 |
| | 3:1 | 3.0 | -- | 1.0 | 0.167 |
| II | 2.5:1 | 2.5 | 1.0 | -- | 0.200 |
| | 1:1 | 1.0 | -- | 1.0 | 0.500 |
| | 3:1 | 3.0 | -- | 1.0 | 0.167 |
| | 5:1 | 5.0 | -- | 1.0 | 0.100 |
| III | 1:3 | 1.0 | -- | 3.0 | 1.500 |
| | 1:2 | 1.0 | -- | 2.0 | 1.000 |
| | 1:1 | 1.0 | -- | 1.0 | 0.500 |
| | 2:1 | 2.0 | -- | 1.0 | 0.250 |
| | 1:1[c] | 1.0 | -- | 1.0[c] | 0.500 |
| IV | 1:1 | 1.0 | 1.0 | -- | 0.500 |
| | 1:1 | 1.0 | -- | 1.0 | 0.500 |

[a] n = 24 animals per treatment.

[b] Volumetric dosage injected subcutaneously at 0 hours.

[c] Two percent CMC substituted for 1 percent CMC used in all other treatments.

Factorial arrangements of treatments in completely randomized designs were used in the first three experiments. Analysis of variance was conducted as described by Steel and Torrie (1960). Tukey's w-procedure, which is based on an experimentwise error rate, was used to locate significant differences between treatment means of ovarian and uterine weights when significant differences were indicated by analysis of variance (Steel and Torrie, 1960). As a matter of convenience to the experimenter, each treatment group of 24 female rats (table 1) was divided randomly into two subgroups of 12. These groups were considered fixed replicates. One subgroup of 12 from each treatment received injections at 0800 hours on day 0 and three animals from it were killed at 24, 48, 72 and 96 hours post-injection. The other subgroups were injected at 1300 hours on day 0 and three animals from each were killed at 24 hour intervals following injection. All treatments were considered fixed variables.

## Experiment I

Factors included in a 2 x 2 x 4 factorial arrangement (table 1) of the data were two carriers (PSS or 1 percent CMC), two levels of hormone (0.0 or 0.5 mg FSH) and four times of sacrifice (24, 48, 72 or 96 hours post-injection). A separate analysis of variance, with 0-hour controls included, was conducted for simple effects between times of sacrifice within CMC alone and between times of sacrifice within PSS alone. This procedure was used in an attempt to evaluate the relative inertness of the carriers and/or the presence of endogenous FSH

activity. Analysis of variance for simple effects between times of sacrifice within FSH in PSS and FSH in CMC were also conducted.

Experiment II

Main factors in a 4 x 4 factorial arrangement (table 1) of the data were four different carriers or ratios of carrier to FSH (PSS at 2.5:1 and CMC at 1:1, 1:3 and 1:5) and four times of sacrifice (24, 48, 72 and 96 hours post-injection).

Experiment III

A 4 x 5 factorial arrangement (table 1) of the data included five different FSH:carrier ratios (FSH in 1 percent CMC in ratios of 1:3, 1:2, 1:1 and 2:1 and FSH in 2 percent CMC at 1:1) and four times of sacrifice (24, 48, 72 and 96 hours post-injection).

Experiment IV

This experiment was conducted as a repetition of two selected treatments from previous experiments to obtain an indication of repeatability of CMC and PSS as carriers for FSH. The treatments were 1 mg FSH:1 ml PSS and 1 mg FSH:1 ml 1 percent CMC (table 1). Simple analysis of variance procedures were used to evaluate both ovarian and uterine growth responses between experiments I, II and IV for FSH in PSS and between experiments II, III and IV for FSH in CMC at a ratio of 1:1.

## Results and Discussion

### Experiment I

Analysis of variance for experiment I (table 2) are presented for primary response (ovarian weight) and secondary response (uterine weight) to treatments. Significant carrier x hormone ($P < .05$) and carrier x time of sacrifice ($P < .05$) interactions suggested further analysis prior to drawing conclusions relative to the significant main effect of hormone level ($P < .01$) on ovarian weight. However, mean ovarian weights for these significant sources of variation (table 3) indicated that FSH tended to stimulate ovarian growth and that 1 percent CMC as a carrier for FSH resulted in heavier ovaries than did the same amount of FSH in PSS.

Several significant interactions as well as main effects of carrier ($P < .01$), hormone ($P < .01$) and time of sacrifice ($P < .05$) were detected for uterine weight (table 2). The tendency for FSH to increase ovarian growth and for CMC to cause an apparent enhancement of this response was reaffirmed by uterine growth in the same animals but at a greater magnitude (table 3).

No significant variation in either ovarian or uterine weights was detected by analysis for time of sacrifice within PSS or CMC (tables 4 and 6). This indicated that either carrier alone did not stimulate ovarian or uterine growth and that endogenous FSH secretions, if present, were not detected during the 96 hour period of this study. The assumption was made for following experiments that both carriers (CMC or PSS) were relatively inactive. It was also assumed that

TABLE 2. ANALYSIS OF VARIANCE FOR CARRIER, HORMONE AND TIME OF SACRIFICE, EXPERIMENT I

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 5.00 | 29.70 |
| Carrier (C) | 1 | 14.34 | 216.60** |
| R x C | 1 | 8.58 | 0.54 |
| Hormone (H) | 1 | 60.33** | 1081.38** |
| R x H | 1 | 18.11 | 5.04 |
| C x H | 1 | 34.68* | 265.36** |
| R x C x H | 1 | 0.25 | 29.70 |
| Time of sacrifice (T) | 3 | 9.54 | 50.61* |
| R x T | 3 | 11.76 | 50.30* |
| C x T | 3 | 31.34* | 43.06* |
| R x C x T | 3 | 4.41 | 29.05 |
| H x T | 3 | 6.62 | 66.06** |
| R x H x T | 3 | 18.32 | 26.84 |
| C x H x T | 3 | 4.98 | 27.56 |
| R x C x H x T | 3 | 20.94 | 28.78 |
| Residual | 64 | 8.37 | 14.12 |

* $P < .05$.
** $P < .01$.

TABLE 3. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR CARRIER, HORMONE, TIME OF SACRIFICE AND SELECTED INTERACTIONS, EXPERIMENT I

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Overall | 15.21 | 26.69 |
| Carrier (C) | | |
| PSS | 14.83 | $25.19^{b}$ |
| 1 percent CMC | 15.60 | $28.20^{a}$ |
| Hormone (H) | | |
| 0.0 mg FSH | $14.42^{b}$ | $23.34^{b}$ |
| 0.5 mg FSH | $16.01^{a}$ | $30.05^{a}$ |
| Time of sacrifice (T) (hours post-injection) | | |
| 24 | 15.68 | $25.94^{ab}$ |
| 48 | 14.64 | $27.88^{a}$ |
| 72 | 15.83 | $27.94^{a}$ |
| 96 | 14.71 | $25.02^{b}$ |
| C x H | | |
| PSS, 0.0 mg FSH | $14.64^{b}$ | $23.50^{c}$ |
| 0.5 mg FSH | $15.02^{ab}$ | $26.89^{b}$ |
| CMC, 0.0 mg FSH | $14.21^{b}$ | $23.18^{c}$ |
| 0.5 mg FSH | $17.00^{a}$ | $33.22^{a}$ |
| C x T | | |
| PSS, 24 | $14.63^{a}$ | $24.43^{b}$ |
| 48 | $13.21^{a}$ | $24.63^{b}$ |
| 72 | $15.62^{a}$ | $26.72^{ab}$ |
| 96 | $15.86^{a}$ | $25.01^{b}$ |
| CMC, 24 | $16.73^{a}$ | $27.46^{ab}$ |
| 48 | $16.07^{a}$ | $31.14^{a}$ |
| 72 | $16.05^{a}$ | $29.16^{ab}$ |
| 96 | $13.56^{a}$ | $25.03^{b}$ |
| H x T | | |
| 0.0 mg FSH, 24 | 14.73 | $22.35^{d}$ |
| 48 | 13.73 | $22.43^{d}$ |
| 72 | 14.57 | $25.03^{cd}$ |
| 96 | 14.67 | $23.56^{d}$ |
| 0.5 mg FSH, 24 | 16.63 | $29.53^{abc}$ |
| 48 | 15.55 | $33.34^{a}$ |
| 72 | 17.10 | $30.85^{ab}$ |
| 96 | 14.75 | $26.48^{bcd}$ |

$^{abcd}$ Means in the same column within variable with different superscript letters differ significantly ($P < .05$).

endogenous FSH secretions did not affect ovarian and uterine growth in female rats which were 26 days of age or younger.

When 0.5 mg of FSH was included with CMC or PSS injections (tables 8 and 10), significant differences due to time of sacrifice were indicated for ovarian weight within FSH in CMC or PSS ($P < .05$) and for uterine weight within FSH in CMC ($P < .01$). Examination of means (tables 5, 7, 9 and 11) suggested that FSH stimulated both ovarian and uterine growth when added to either PSS or CMC and that CMC tended to increase these growth responses. FSH with CMC as the carrier resulted in heavier weights than the same amount of FSH in PSS (tables 9 and 11). However, error mean squares (tables 4, 6, 8 and 10) indicate that as the degree of stimulation increased variability also increased slightly.

These data suggest the following preliminary conclusions: (1) PSS or 1 percent CMC administered as single injections to immature female rats did not stimulate growth of ovarian or uterine tissues, (2) Endogenous secretions of FSH, if present, were not detected during the experimental period, (3) FSH at 0.5 mg per single injection had a stimulatory effect on the reproductive organs of immature rats and (4) CMC tended to enhance the activity of FSH injections.

## Experiment II

Two treatments from experiment I, FSH in PSS and FSH in 1 percent CMC (3:1), were repeated in this experiment. In addition, FSH in CMC (1:1 and 5:1) were added in order to study the effects of

TABLE 4. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE WITHIN PSS ONLY, EXPERIMENT I

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 0.68 | 29.01 |
| Time of sacrifice (T) | 4 | 9.90 | 6.74 |
| R x T | 4 | 22.01 | 14.34 |
| Residual | 20 | 7.70 | 6.76 |

TABLE 5. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR TIME OF SACRIFICE WITHIN PSS ONLY, EXPERIMENT I

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Time of sacrifice (hours post-injection) | | |
| 0 | 12.63 | 23.18 |
| 24 | 14.15 | 22.62 |
| 48 | 13.45 | 22.30 |
| 72 | 15.07 | 24.65 |
| 96 | 15.88 | 24.43 |

TABLE 6. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE WITHIN CMC ONLY, EXPERIMENT I

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 1.83 | 1.37 |
| Time of sacrifice (T) | 4 | 5.78 | 10.16 |
| R x T | 4 | 6.40 | 11.34 |
| Residual | 20 | 5.00 | 7.88 |

TABLE 7. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR TIME OF SACRIFICE WITHIN CMC ONLY, EXPERIMENT I

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Time of sacrifice (hours post-injection) | | |
| 0 | 12.63 | 23.18 |
| 24 | 15.32 | 22.08 |
| 48 | 14.00 | 22.55 |
| 72 | 14.07 | 25.40 |
| 96 | 13.45 | 22.68 |

TABLE 8. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE WITHIN FSH IN PSS, EXPERIMENT I

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 6.44 | 2.24 |
| Time of sacrifice (T) | 4 | 16.14* | 25.06 |
| R x T | 4 | 2.64 | 13.34 |
| Residual | 20 | 5.42 | 14.97 |

* $P < .05$.

TABLE 9. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR TIME OF SACRIFICE WITHIN FSH IN PSS, EXPERIMENT I

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Time of sacrifice (hours post-injection) | | |
| 0 | 12.63[a] | 23.18 |
| 24 | 15.12[a] | 26.23 |
| 48 | 12.97[a] | 26.95 |
| 72 | 16.17[a] | 28.78 |
| 96 | 15.83[a] | 15.58 |

[a] Means in same column with different superscript letters differ significantly ($P < .05$).

TABLE 10. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE WITHIN FSH IN CMC (3:1), EXPERIMENT I

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 31.41 | 16.28 |
| Time of sacrifice (T) | 4 | 45.02* | 235.90** |
| R x T | 4 | 15.25 | 76.87* |
| Residual | 20 | 11.69 | 21.58 |

* $P < .05$.
** $P < .01$.

TABLE 11. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR TIME OF SACRIFICE WITHIN FSH IN CMC (3:1), EXPERIMENT I

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Time of sacrifice (hours post-injection) | | |
| 0 | 12.63[a] | 23.18[c] |
| 24 | 18.15[a] | 32.83[ab] |
| 48 | 18.13[a] | 39.73[a] |
| 72 | 18.03[a] | 32.93[ab] |
| 96 | 13.67[a] | 27.38[bc] |

[abc] Means in same column with different superscript letters differ significantly ($P < .05$).

hormone-to-carrier ratio on ovarian and uterine growth. Analysis of variance (table 12) indicated significant differences in ovarian weights due to time of sacrifice ($P < .01$) and carrier ($P < .05$).

Tukey's w-procedure applied to mean ovarian weights (table 13) indicated the four times of sacrifice at 24-hour intervals after injection were all different ($P < .05$) from each other with a definite peak response at 24 hours post-injection. Ovarian growth was greater from injections of FSH:CMC (1:1) than the other treatments. However, this response was not significantly different from either FSH in PSS or FSH in CMC (3:1).

Significant replicate (R) x carrier (C), time (T) x carrier and R x T x C interactions ($P < .01$) limit interpretation of significant main effects ($P < .01$) on uterine growth. However, examination of C x T means (table 13) indicates a maximal response to FSH in PSS at the 24-hour post-injection sacrifice as compared to a 48-hour maximum for all ratios of FSH in CMC. Maximal uterine growth in response to FSH in CMC tended to be greater than FSH in PSS with the heaviest weights observed from stimulation by FSH in CMC (1:1). This apparent tendency of CMC to delay and maximize uterine growth response to exogenous FSH may be due to the colloidal and/or the ion-exchange properties of CMC (Sober and Peterson, 1958; Harper, 1965; Hercules Inc., 1971). Either of these properties may tend to slow the release of active hormone from the site of injection. However, an accurate evaluation of the physiological and chemical mechanisms involved is beyond the scope of this study as designed and conducted.

TABLE 12. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE AND CARRIER, EXPERIMENT II

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 8.88 | 37.88 |
| Time of sacrifice (T) | 3 | 341.43** | 1408.31** |
| R x T | 3 | 3.84 | 34.05 |
| Carrier (C) | 3 | 31.75* | 1093.45** |
| R x C | 3 | 14.00 | 144.16** |
| T x C | 9 | 8.76 | 94.84** |
| R x T x C | 9 | 14.69 | 78.66** |
| Residual | 64 | 8.53 | 17.96 |

* $P < .05$.
** $P < .01$.

TABLE 13. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR TIME OF SACRIFICE, CARRIER AND THEIR INTERACTIONS, EXPERIMENT II

| Variable | | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|---|
| Overall | | 15.94 | 33.72 |
| Time of sacrifice (T) (hours post-injection) | | | |
| 24 | | $20.12^{a}$ | $38.34^{a}$ |
| 48 | | $17.63^{b}$ | $41.45^{a}$ |
| 72 | | $14.63^{c}$ | $30.57^{b}$ |
| 96 | | $11.39^{d}$ | $24.50^{c}$ |
| Carrier (C) | | | |
| FSH:PSS | | $15.45^{ab}$ | $27.92^{c}$ |
| FSH:CMC (1:1) | | $17.51^{a}$ | $43.30^{a}$ |
| FSH:CMC (3:1) | | $16.00^{ab}$ | $33.25^{b}$ |
| FSH:CMC (5:1) | | $14.82^{b}$ | $30.40^{c}$ |
| C x T | | | |
| FSH:PSS, | 24 | 18.77 | $34.62^{bcde}$ |
| | 48 | 15.52 | $28.30^{defg}$ |
| | 72 | 15.58 | $27.62^{efg}$ |
| | 96 | 11.92 | $21.15^{g}$ |
| FSH:CMC (1:1), | 24 | 22.57 | $49.28^{a}$ |
| | 48 | 18.82 | $56.30^{a}$ |
| | 72 | 15.58 | $37.10^{bc}$ |
| | 96 | 13.07 | $30.52^{cdef}$ |
| FSH:CMC (3:1), | 24 | 20.55 | $37.08^{bc}$ |
| | 48 | 17.87 | $41.15^{b}$ |
| | 72 | 14.08 | $28.93^{cdefg}$ |
| | 96 | 11.32 | $25.82^{fg}$ |
| FSH:CMC (5:1), | 24 | 18.58 | $32.38^{cdef}$ |
| | 48 | 18.30 | $36.72^{bcd}$ |
| | 72 | 13.28 | $28.65^{cdefg}$ |
| | 96 | 9.10 | $20.52^{g}$ |

$^{abcdefg}$ Means in same column within variable with different superscript letters differ significantly ($P < .05$).

## Experiment III

In an attempt to identify an optimum ratio of FSH to CMC, the 1:1 ratio which caused the greatest stimulation in growth of the reproductive tract of immature female rats in experiment II was repeated. Two lower and one higher ratio (1:2, 1:3 and 2:1) of FSH to CMC were added. At ratios of 1 mg FSH to 3 ml of 1 percent CMC, relatively large injections were required (table 1) to provide the selected constant of 0.5 mg FSH per dose. Therefore, an additional treatment of 1 mg FSH in 1 ml of 2 percent CMC (theoretically equivalent to 1 mg FSH in 2 ml of 1 percent CMC) was added to this experiment.

Significant interactions were not detected by analysis of variance (table 14) which allows direct inferences concerning highly significant main effects ($P < .01$) on both ovarian and uterine growth. Tukey's w-procedure was used to locate significant differences between ovarian and uterine weights for time of sacrifice and for hormone: carrier ratios (table 15). The greatest growth response in primary and secondary parameters occurred at 48 hours post-injection ($P < .05$). The similarity between ovarian and uterine growth curves in this experiment was different than observed in experiment II where maximum uterine growth was observed at approximately 24 hours after the maximum ovarian response. A time lapse between maximum ovarian and uterine growth as primary and secondary reactions, respectively, might be expected since uterine growth is considered a result of estrogen from growing ovarian follicles. However, the apparent consistent responses of these parameters to FSH in CMC and the relatively long

TABLE 14. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE AND HORMONE:CARRIER RATIOS, EXPERIMENT III

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 20.75 | 33.71 |
| Time of sacrifice (T) | 3 | 323.67** | 4129.83** |
| R x T | 3 | 32.75 | 144.70 |
| Carrier (C) | 4 | 66.04** | 925.26** |
| R x C | 4 | 12.31 | 90.16 |
| T x C | 12 | 13.44 | 125.76 |
| R x T x C | 12 | 5.75 | 84.83 |
| Residual | 80 | 13.65 | 69.49 |

** $P < .01$.

TABLE 15. MEANS OF OVARIAN AND UTERINE WEIGHT FOR TIME OF SACRIFICE AND CARRIER, EXPERIMENT III

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Overall | 19.26 | 50.39 |
| Time of sacrifice (hours post-injection) | | |
| 24 | $19.95^{b}$ | $45.57^{bc}$ |
| 48 | $23.32^{a}$ | $67.22^{a}$ |
| 72 | $18.34^{b}$ | $48.54^{b}$ |
| 96 | $15.44^{c}$ | $40.25^{c}$ |
| Carrier | | |
| FSH:1 percent CMC (1:3) | $20.43^{a}$ | $56.25^{a}$ |
| (1:2) | $20.73^{a}$ | $55.11^{a}$ |
| (1:1) | $18.36^{ab}$ | $45.77^{b}$ |
| (2:1) | $16.79^{b}$ | $42.11^{b}$ |
| FSH:2 percent CMC (1:1) | $20.00^{a}$ | $52.84^{a}$ |

$^{abc}$ Means in the same column within variable with different superscript letters differ significantly ($P < .05$).

(24 hour) interval between observations may account for failure to detect a time lag between ovarian and uterine weight peaks for the ratios of FSH to CMC used in this experiment.

An apparent plateau of mean responses to FSH in 1 percent CMC (1:2 and 1:3) and FSH in 2 percent CMC (1:1) was indicated (table 15). This suggests that the optimum FSH-to-carrier ratio for the type of CMC used in this study involving immature female rats was 1 mg FSH to 2 ml or more of 1 percent CMC.

## Experiment IV

The apparent inconsistent responses to FSH in PSS as compared to FSH in CMC in previous experiments suggested further investigation of repeatability of these treatments. FSH in PSS in experiment I resulted in the heaviest ovarian and uterine weights at 72 hours post-injection (table 3), but the same treatment in experiment II resulted in the heaviest weights at the 24 hour observation (table 13). On the other hand, ovarian and uterine growth curves in response to FSH in CMC suggested more similarity between experiments (tables 11 and 13).

Treatments selected for repetition in this experiment were FSH in PSS and FSH in CMC at a ratio of 1:1 (table 1). Analysis of variance between experiments I, II and IV for FSH in PSS (table 16) indicated highly significant interactions. However, these same interactions were not significant in comparisons between experiments II, III and IV for FSH in CMC at the 1:1 ratio (table 18). Time of sacrifice was a highly significant ($P < .01$) source of variation in ovarian and uterine weights for FSH in PSS and FSH in CMC (1:1).

TABLE 16. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE AND EXPERIMENTS I, II AND IV WITHIN FSH IN PSS

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 3.21 | 84.07* |
| Experiment (E) | 2 | 2.53 | 81.85* |
| R x E | 2 | 40.70** | 25.81 |
| Time of sacrifice (T) | 3 | 27.99** | 96.82** |
| R x T | 3 | 16.56 | 7.15 |
| E x T | 6 | 22.80** | 98.25** |
| R x E x T | 6 | 3.84 | 43.12* |
| Residual | 48 | 6.46 | 16.50 |

* $P < .05$.
** $P < .01$.

TABLE 17. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR EXPERIMENTS I, II AND IV, TIME OF SACRIFICE AND THEIR INTERACTIONS WITHIN FSH IN PSS

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Overall | 15.38 | 28.43 |
| Experiment | | |
| I | 15.02 | $26.89^{b}$ |
| II | 15.44 | $27.92^{ab}$ |
| IV | 15.66 | $30.48^{a}$ |
| Time of sacrifice (hours post-injection) | | |
| 24 | $17.17^{a}$ | $31.09^{a}$ |
| 48 | $14.74^{b}$ | $29.11^{ab}$ |
| 72 | $15.22^{ab}$ | $27.97^{ab}$ |
| 96 | $14.37^{b}$ | $25.54^{b}$ |
| E x T | | |
| Experiment I, 24 | $15.12^{abc}$ | $26.23^{bc}$ |
| 48 | $12.97^{bc}$ | $26.95^{abc}$ |
| 72 | $16.17^{abc}$ | $28.78^{abc}$ |
| 96 | $15.83^{abc}$ | $25.58^{bc}$ |
| Experiment II, 24 | $18.77^{a}$ | $34.62^{a}$ |
| 48 | $15.52^{abc}$ | $28.30^{abc}$ |
| 72 | $15.58^{abc}$ | $27.62^{abc}$ |
| 96 | $11.92^{c}$ | $21.15^{a}$ |
| Experiment IV, 24 | $17.63^{ab}$ | $32.43^{ab}$ |
| 48 | $15.73^{abc}$ | $32.08^{ab}$ |
| 72 | $13.90^{abc}$ | $27.50^{abc}$ |
| 96 | $15.37^{abc}$ | $29.88^{ab}$ |

$^{abc}$ Means in the same column within variable with different superscript letters differ significantly ($P < .05$).

TABLE 18. ANALYSIS OF VARIANCE FOR TIME OF SACRIFICE AND EXPERIMENTS II, III AND IV WITHIN FSH IN CMC (1:1)

| Source | d.f. | Mean square ovarian wt | Mean square uterine wt |
|---|---|---|---|
| Replicate (R) | 1 | 0.06 | 3.21 |
| Experiment (E) | 2 | 6.08 | 141.15 |
| R x E | 2 | 11.87 | 25.73 |
| Time of sacrifice (T) | 3 | 191.34** | 1883.66** |
| R x T | 3 | 29.93* | 129.32 |
| E x T | 6 | 11.13 | 83.14 |
| R x E x T | 6 | 3.16 | 29.83 |
| Residual | 48 | 10.59 | 75.05 |

* $P < .05$.
** $P < .01$.

Differences between experiments were significant ($P < .05$) only in comparison of uterine weights in response to FSH in PSS.

These results indicate that ovarian and uterine growth of immature female rats in response to 0.5 mg FSH in PSS tended to be less repeatable than when the same amount of FSH was injected with CMC as the carrier at 1 mg FSH to 1 ml of 1 percent CMC (tables 17 and 19). However, the error mean square for comparisons between experiments with FSH in CMC (table 18) was nearly five times as large as for FSH in PSS (table 16) and may account for failure to detect other significant sources of variation. These comparisons apparently reaffirm the observation in experiment I that the variability in

TABLE 19. MEANS OF OVARIAN AND UTERINE WEIGHTS FOR EXPERIMENTS II, III AND IV, TIME OF SACRIFICE AND THEIR INTERACTIONS WITHIN FHS IN CMC (1:1)

| Variable | Ovarian weight (mg) | Uterine weight (mg) |
|---|---|---|
| Overall | 18.09 | 45.74 |
| Experiment | | |
| II | 17.51 | 43.30 |
| III | 18.36 | 45.77 |
| IV | 18.40 | 48.15 |
| Time of sacrifice (hours post-injection) | | |
| 24 | 21.59[a] | 46.12[b] |
| 48 | 19.84[a] | 59.60[a] |
| 72 | 16.63[b] | 41.78[b] |
| 96 | 14.29[b] | 35.44[c] |
| E x T | | |
| Experiment II, 24 | 22.57 | 49.28 |
| 48 | 18.82 | 56.30 |
| 72 | 15.58 | 37.10 |
| 96 | 13.07 | 30.52 |
| Experiment III, 24 | 19.60 | 40.92 |
| 48 | 20.98 | 61.70 |
| 72 | 17.27 | 43.55 |
| 96 | 15.58 | 36.90 |
| Experiment IV, 24 | 22.60 | 48.17 |
| 48 | 19.73 | 60.80 |
| 72 | 17.05 | 44.70 |
| 96 | 14.22 | 38.92 |

[abc] Means in the same column within variable with different superscript letters differ significantly ($P < .05$).

uterine weight tended to increase directly with degree of stimulation. The same tendency was not as distinct for ovarian weights where relative responses were not as large (tables 16 and 18). The tendency for increased ovarian stimulation by gonadotropins to be accompanied by increased variability has also been reported for cattle (Gordon et al., 1962; Mills and Vincent, 1969). Intra-uterine fluid accumulations tended to vary in highly stimulated uteri and may account for part of the apparent increase in variability observed in this secondary response.

## Summary

A series of four experiments involving 384 immature female rats were conducted to compare physiological saline (PSS) and carboxymethylcellulose (CMC) as carriers for follicle stimulating hormone (FSH) and to evaluate quantitative relationships of FSH and CMC.

One percent CMC as a carrier resulted in heavier ovarian and uterine weights than did the same quantity (0.5 mg) of FSH in PSS. The apparent optimum ratio of FSH:CMC in the rat was 1 mg FSH to 2 ml or more of 1 percent CMC solution. There was also an indication that ovarian and uterine stimulation by FSH in PSS was less repeatable than FSH in CMC.

These data do not permit conclusions relative to the mechanisms involved in the apparent enhancement of FSH activity by CMC.

# SECTION II

# EFFECTS OF LEVEL AND DAY OF INJECTION OF FSH IN CMC ON OVARIAN STIMULATION IN THE BOVINE

## Introduction

Twinning in beef cattle should lead to increased efficiency and economic advantages for many cow-calf producers. It is anticipated that a procedure which would result in a high incidence of twin calves might be most readily incorporated into management programs which are relatively intensive.

Exogenous gonadotropins, follicle stimulating hormone (FSH) and pregnant mare serum (PMS) have been used to obtain controlled superovulation (Bellows, Anderson and Short, 1969; Schilling and Holm, 1963; Laster et al., 1971) and controlled multiple births (Turman et al., 1971). It has been suggested that less variability in follicular development resulted from the use of FSH than PMS (Dziuk et al., 1958).

Bellows et al. (1969) reported an average of 2.1 corpora lutea (CL) per heifer injected with a total dose of 6.26 mg of FSH. This dosage was given by twice daily injections of 0.625 mg FSH on days 8 through 12 of an 11-day feeding of medroxyprogesterone acetate (MAP). However, the relatively large number of injections involved tends to limit the probable commercial usefulness of this procedure. Carboxymethylcellulose (CMC) has been suggested as a diluent which might reduce the number of injections required by prolonging the absorption rate of FSH (Vincent, 1970).

The purpose of this study was to evaluate the effects of dosage and day of injection of FSH (carried in CMC) on ovarian stimulation in synchronized and nonsynchronized beef heifers. The effects of an

estradiol valerate (EV) injection to induce CL regression on ovarian response to FSH were also studied.

## Materials and Methods

### General

Follicle stimulating hormone (FSH-P)[4] was used as the source of gonadotropin in these experiments. Carboxymethylcellulose (CMC)[5] in a 1 percent solution was used as the carrier for all FSH injections. The lyophilized FSH was activated by adding 2 to 5 ml of sterile CMC followed by further dilution to desired dosages. Final dilutions were refrigerated for a minimum of 1 hour and a maximum of 4 hours prior to injection.

Parameters of ovarian response to treatments were number of ovulation sites, follicles $\geq$10 mm and fetuses. These data, measured in small, discrete numbers, were transformed by $\sqrt{X + 0.5}$ prior to analysis of variance (Steel and Torrie, 1960). All treatments were considered fixed.

### Experiment V

Phase I. Forty-eight, yearling, commercial Hereford heifers, weighing 215 to 298 kg in early April, 1970, were used in this study.

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[4]FSH-P (Follicle stimulating hormone-pituitary), Armour-Baldwin Laboratories, Omaha, Nebraska. Supplied as a lyophilized powder obtained from pituitary glands of domestic animals. Each 10 cc vial is listed to contain the equivalent of 50 mg Armour Standard FSH units and levels reported are based on this value.

[5]CMC (sodium carboxymethylcellulose), Hercules Powder Company, Wilmington, Delaware. Hercules CT grade, 75 percent sodium carboxymethylcellulose, balance salt of reaction, Lot #7838.

All heifers were managed as a single lot throughout the experiment. Average to good quality alfalfa hay plus a limited (1 kg/day) grain ration of one-third oats and two-thirds corn was group fed to produce about 0.45 kg per head per day gain.

Individual estrous cycles were established prior to and during treatment by morning and evening observations with the aid of two vasectomized bulls. The bulls were allowed access to the heifers during periods of observation only. Those heifers standing for the bulls or for other heifers were recorded. Positive estrus was not observed in 12 of the 48 heifers prior to assignment to treatment groups.

The heifers were lotted to six weight-stratified groups of eight each with two of those not showing prior heat assigned to each group. Treatments were then assigned to each group randomly (table 20).

Synchronization of estrus was obtained by the addition of 1 mg per head of melengesterol acetate (MGA)[6] to the daily grain ration (Zimbelman and Smith, 1966) for a period of 14 days. One-half of the group (table 20) received an intramuscular injection of estradiol valerate (EV)[7] on day 2 of MGA feeding as recommended by Wiltbank and

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[6]MGA (melengesterol acetate), MGA-100 Premix. Each pound containing 100 mg melengesterol acetate (U-21240), Lot #40,005-26. The Upjohn Company, Kalamazoo, Michigan.

[7]Delestrogen, containing 20 mg estradiol valerate per cc, E. R. Squibb and Sons, Inc., New York, New York.

TABLE 20. EXPERIMENTAL DESIGN FOR EXPERIMENT V

| Treatment | No. of animals | | EV (mg)[a] | Day of FSH injection[b] |
|---|---|---|---|---|
| | Phase I | Phase II | | |
| Control[c] | 0 | 7 | -- | -- |
| 1 | 8 | 7 | 5.0 | 10 |
| 2 | 8 | 7 | 5.0 | 12 |
| 3 | 8 | 7 | 5.0 | 14 |
| 4 | 8 | 7 | 0.0 | 10 |
| 5 | 8 | 7 | 0.0 | 12 |
| 6 | 8 | 7 | 0.0 | 14 |

[a] Estradiol valerate injected intramuscularly on day 2 of MGA feeding.

[b] A single, intramuscular injection of 7 mg FSH in 2 ml (phase I) or 3 ml (phase II) of 1 percent CMC was given on day 10, 12 or 14 of MGA feeding.

[c] Control, included in phase II only, fed MGA and injected with 2 or 3 ml of 1 percent CMC on day 10, 12 or 14.

Kasson (1968). FSH was given as a single, intramuscular injection of 7 mg in 2 ml of 1 percent CMC to each animal on days 10, 12 or 14 of MGA feeding. Ovulation rate and coincident follicular development (follicles $\geq$10 mm) were determined by high lumbar laparotomy (Casida, 1960) at 72 to 96 hours after the observed onset of post-treatment estrus.

Phase II. Heifers from phase I, plus one which had been purchased as an extra, were used in phase II of this experiment. A period of 10 weeks elapsed between the initiation of treatments in phases I and II. During this interval, good quality summer pasture was provided for three weeks prior to returning the heifers to drylot where they were managed as described in phase I.

The 49 heifers were assigned to seven treatment groups of seven heifers each, stratified on the basis of previous treatments. Two heifers were assigned to the same treatment they had received in phase I. The remaining six heifers from each treatment in phase I were then assigned at the rate of one each to the other six treatments. Hence, heifers from all previous treatments were represented in each new group. Treatments were a repetition of those used in phase I with the addition of a control group (table 20) which received volumetrically equivalent injections of CMC.

All procedures for feeding, detection and synchronization of estrus and administration of exogenous hormones were conducted in the same manner as for phase I with one exception. In this phase, all heifers were artificially inseminated at the first post-treatment estrus. A second laparotomy was performed (incision adjacent to first) at 72 to 96 hours following insemination to obtain ovarian stimulation data. All heifers were slaughtered at 31 to 44 days post-insemination. Reproductive tracts were recovered and examined for number of active corpora lutea (CL) and live embryos.

## Experiment VI

Phase I. Fifty yearling, Hereford-Angus crossbred heifers were purchased for this study. The average purchase weight of 258 kg ranged from 229 to 284 kg in late March, 1971. A ration of good quality alfalfa hay and/or corn silage periodically plus approximately 1.8 kg per head per day of one-third oats and two-thirds corn resulted in an average daily gain of approximately 0.57 kg per head.

Individual estrous cycles were established during April and May by twice daily (morning and evening) observations for estrual behavior. Only those definitely standing for other heifers were recorded and 4 of the 50 were not observed in estrus during this period. Beginning May 31, daily checks were increased to early morning, noon and late evening to provide base dates for treatments (day 0 = day of first observation in estrus). As groups of five were observed in estrus, they were assigned randomly to treatment groups (table 21) forming a stratification on day of estrus.

FSH injections with CMC as the carrier at a ratio of 1 mg FSH to 2 ml 1 percent CMC were given subcutaneously. Control heifers received volumetric equivalents of CMC. Ovarian stimulation data (number of ovulation sites and follicles ≥10 mm) were determined as described in experiment V.

TABLE 21. EXPERIMENTAL DESIGN FOR EXPERIMENT VI, PHASE I

| Treatment | No. of animals | Total dose FSH (mg)[a] | FSH (mg)[a] | |
|---|---|---|---|---|
| | | | Day 15[b] | Day 17[b] |
| Control[c] | 10 | -- | -- | -- |
| 1 | 10 | 7.0 | 7.0 | -- |
| 2 | 10 | 7.0 | 3.5 | 3.5 |
| 3 | 10 | 10.0 | 10.0 | -- |
| 4 | 10 | 10.0 | 5.0 | 5.0 |

[a] Subcutaneous injections of FSH (1 mg FSH:2 ml 1 percent CMC).
[b] Day 0 = observed onset of estrus.
[c] Injected with volumetric equivalents of CMC on day 15 and/or 17.

Phase II. Five of the 50 heifers used in phase I were selected at random and removed for use in another trial. The remaining 45 heifers were assigned to five different treatment groups (table 22) by the procedure described in phase I. The control group was reduced to five. A period of 12 weeks between initiation of treatments in the two phases was allowed for recovery from surgical stresses. All treatment procedures were conducted as described in phase I.

In order to obtain a measure of fertility, all heifers were inseminated artificially at the first and also second post-treatment estrus, if expressed. The number of fetuses and active CL were determined by laparotomy at 43 to 57 days following the last insemination.

TABLE 22. EXPERIMENTAL DESIGN FOR EXPERIMENT VI, PHASE II

| Treatment | No. of animals | FSH (mg)[a] Day of injection first estrous cycle[b] 15 | 17 | 16 | 18 | Day of injection second estrous cycle[c] 15 | 17 | 16 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Control[d] | 5 | -- | -- | -- | -- | -- | -- | -- | -- |
| 1 | 10 | 5.0 | 5.0 | -- | -- | -- | -- | -- | -- |
| 2 | 10 | 5.0 | 5.0 | -- | -- | 5.0 | 5.0 | -- | -- |
| 3 | 10 | -- | -- | 5.0 | 5.0 | -- | -- | -- | -- |
| 4 | 10 | -- | -- | 5.0 | 5.0 | -- | -- | 5.0 | 5.0 |

[a] Subcutaneous injections of FSH (1 mg FSH:2 ml 1 percent CMC).
[b] Day 0 = observed onset of estrus.
[c] Day 0 = observed onset of first post-treatment estrus with artificial insemination on day 1.
[d] Injected with volumetric equivalents of CMC.

## Results and Discussion

### Experiment V

This trial was conducted to study the effects of day of injection of FSH (carried in CMC) in beef heifers with synchronized estrous cycles and to study the effects of EV-induced CL regression on ovarian response to FSH (table 20).

Estradiol valerate and day of FSH injection were not significant sources of variation in ovulation rate or total stimulation (ovulation sites plus follicles $\geq$10 mm) in phase I. However, a significant EV x day of injection ($P<.05$) interaction limited positive conclusions concerning the effects of treatments on ovulation rate (table 23). Nontransformed treatment means (table 24) for phase I indicated no tendency for the formation of trends in ovulation rate from treatments applied. Ovulation rate was greatest from 7 mg of FSH given on day 10 of MGA feeding to heifers receiving EV injections and from the same amount of FSH injected on day 14 to heifers which did not receive EV (table 24). On the other hand, an average of less than one ovulation site per heifer was observed in phase I for both 0.0 and 5.0 mg EV with FSH given on day 12 and 14, respectively. Ovulation sites were not observed at laparotomy in 8 of the 48 heifers studied.

The relatively high incidence of anovulatory heifers suggested that some of them may not have reached puberty. Twelve of the 48 were not detected in estrus prior to treatment. Also, their sires

TABLE 23. ANALYSIS OF VARIANCE FOR EXPERIMENT V

| Source | d.f. | Mean square ovulation rate[a] | Mean square total stimulation[b] |
|---|---|---|---|
| | Phase I | | |
| Estradiol valerate (E) | 1 | 0.0106[c] | 0.1015 |
| Day of FSH injection (D) | 2 | 0.0573 | 0.0614 |
| E x D | 2 | 0.2897* | 0.0804 |
| Residual | 42 | 0.0758 | 0.1503 |
| | Phase II | | |
| Treatment | 6 | 0.1366 | 0.2201 |
| Residual | 42 | 0.0863 | 0.1900 |

* $P < .05$.

[a] Computed from transformed values of observed CL at laparotomy.

[b] Computed from transformed values of observed CL plus follicles $\geq$10 mm at laparotomy.

[c] Tabular values computed from data transformed to $\sqrt{X + 0.5}$ for analysis.

TABLE 24. DISTRIBUTION OF OVULATION SITES, EXPERIMENT V

| Treatments | | Zero CL[a] | | Single CL[a] | | Multiple CL[a,b] | | Estradiol valerate[c] | | Day of FSH[c] | | EV x day[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phase I | | | % | | % | | % | | $\bar{x}$ | | $\bar{x}$ | | $\bar{x}$ |
| 5.0 mg EV, FSH day | 10 | 1/8 | 12.5 | 4/8 | 50.0 | 3/8 | 37.5 | 25/24 | 1.04 | 19/16 | 1.19 | 11/8 | 1.38 |
| | 12 | 1/8 | 12.5 | 6/8 | 75.0 | 1/8 | 12.5 | | | 15/16 | 0.94 | 9/8 | 1.13 |
| | 14 | 4/8 | 50.0 | 3/8 | 37.5 | 1/8 | 12.5 | | | 16/16 | 1.00 | 5/8 | 0.63 |
| 0.0 mg EV, FSH day | 10 | 0/8 | 0.0 | 8/8 | 100.0 | 0/8 | 0.0 | 25/24 | 1.04 | | | 8/8 | 1.00 |
| | 12 | 2/8 | 25.0 | 6/8 | 75.0 | 0/8 | 0.0 | | | | | 6/8 | 0.75 |
| | 14 | 0/8 | 0.0 | 6/8 | 75.0 | 2/8 | 25.0 | | | | | 11/8 | 1.38 |
| Overall | | 8/48 | 16.7 | 33/48 | 68.7 | 7/48 | 14.6 | | | | | 50/48 | 1.04 |
| Phase II | | | | | | | | | | | | | |
| Control | | 1/7 | 14.3 | 6/7 | 85.7 | 0/7 | 0.0 | | | | | 6/7 | 0.86 |
| 5.0 mg EV, FSH day | 10 | 1/7 | 14.3 | 6/7 | 85.7 | 0/7 | 0.0 | 19/21 | 1.07 | 15/14 | 1.07 | 6/7 | 0.86 |
| | 12 | 3/7 | 42.9 | 4/7 | 57.1 | 0/7 | 0.0 | | | 16/14 | 1.14 | 4/7 | 0.57 |
| | 14 | 1/7 | 14.3 | 4/7 | 57.1 | 2/7 | 28.6 | | | 17/14 | 1.21 | 9/7 | 1.29 |
| 0.0 mg EV, FSH day | 10 | 0/7 | 0.0 | 5/7 | 71.4 | 2/7 | 28.6 | 29/21 | 1.38 | | | 9/7 | 1.29 |
| | 12 | 1/7 | 14.3 | 3/7 | 42.9 | 3/7 | 42.9 | | | | | 12/7 | 1.71 |
| | 14 | 1/7 | 14.3 | 4/7 | 57.1 | 2/7 | 28.6 | | | | | 8/7 | 1.14 |
| Overall | | 8/49 | 16.3 | 32/49 | 65.3 | 9/49 | 18.4 | | | | | 54/49 | 1.10 |

[a] Nontransformed data showing frequency and percent ovulation types observed at laparotomy.
[b] Range of multiple ovulations = 2 to 3 per heifer.
[c] Frequency of ovulation sites per group and average number of ovulations per heifer. (Averages represent treatment means of nontransformed data.)

and ages were unknown and some of them were relatively small (9 weighed <250 kg at the beginning of treatment). Arije and Wiltbank (1971) reported that sires and day of birth within year significantly affected age and weight at puberty. The occurrence of puberty was reported at an average weight of 250.9 kg and 436.4 days of age in 235 Hereford heifers studied in two consecutive years.

However, only three of the eight heifers in this phase which did not show ovulation sites at laparotomy had not been detected in estrus prior to treatment and all of the eight did exhibit definite estrual behavior prior to laparotomy. Hence, it is doubtful that the non-ovulatory heifers observed can be considered due entirely to prepuberal heifers. Rather, the errotic responses observed were more likely due to "animal differences." Dziuk et al. (1958) reported considerable variation in ovarian response between cattle treated alike. This observation has been reaffirmed by Gordon, Williams and Edwards (1962), Hafez et al. (1965) and Sitton, Smith and Vincent (1972). The term animal differences is ambiguous but implies genetic differences in ovarian response to stimulation with exogenous gonadotropins which have been identified for rats (Rollins and Cole, 1952), mice (Zarrow, Christenson and Eleftheriou, 1971; Wolfe, 1971) and sheep (Wallace, 1954; Gordon, 1958). Environmental influences were considered as uniform as possible for cattle managed in a single lot and group fed.

In view of the apparently high incidence of anovulatory heifers observed in phase I, a control group was included in phase II (table 20) in addition to repetition of the six treatments used in the

previous trial. Treatments were not detected as significant sources of variation for either ovulation rate or total stimulation (table 23). Although nonsignificant, there was an indication in this phase (table 24) that EV tended to reduce the average number of ovulation sites observed.

Relatively wide variations in magnitude as well as direction of response to treatments were apparent in both phases of this experiment. Ovulation rate, computed from nontransformed data, was 2.2 times as great from heifers which did not receive EV than from those that did when FSH injections (7 mg FSH in 2 ml CMC) were given on day 14 in phase I (table 24). On the other hand, ovulation rate was three times as great from heifers not receiving EV than from those that did when FSH was injected on day 12 in phase II (table 24). Orthogonal comparisons (table 25) indicate that these differences were significant ($P<.05$). However, conclusions as to whether responses were due to a depressing effect of EV or an extrastimulatory effect of FSH given on day 12 or 14 of MGA feeding cannot be made on the basis of these data.

Since there was a relatively short (10 weeks) interval between phases in this trial, like treatments from the two phases (table 20) were compared. Phase x EV x day of injection was the only significant ($P<.01$) source of variation in ovulation rate, and no significant effects on total ovarian stimulation were detected (table 26). The inclusion or omission of 5.0 mg estradiol valerate injections approached significance ($P<.10$), suggesting an apparent negative effect on ovulation rate (tables 26 and 27). Significant interactions

TABLE 25. MEAN SQUARES[a] FROM ORTHOGONAL COMPARISONS OF OVARIAN DATA, EXPERIMENT V

| Comparison no. | Source of variation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| Measurement | Control vs. rest | Day 14 vs. day 10 + 12 | Day 10 vs. day 12 | EV vs. no EV day 10 | EV vs. no EV day 12 | EV vs. no EV day 14 | Error |
| d.f. | (1) | (1) | (1) | (1) | (1) | (1) | 42 |
| Phase I | | | | | | | |
| Ovulation rate[b] | -- | 0.0191[a] | 0.0954 | 0.0442 | 0.0846 | 0.4612* | 0.0758 |
| Total stimulation[c] | -- | 0.0001 | 0.1227 | 0.0353 | 0.0103 | 0.2167 | 0.1503 |
| Phase II | | | | | | | |
| Ovulation rate | 0.0503 | 0.0200 | 0.0038 | 0.1081 | 0.6315* | 0.0064 | 0.0863 |
| Total stimulation | 0.2634 | 0.2301 | 0.0169 | 0.2061 | 0.5970 | 0.0073 | 0.1900 |

* $P < .05$.

[a] Tabular values computed from data transformed to $X + 0.5$ for analysis.

[b] Computed from transformed values of observed CL at laparotomy.

[c] Computed from transformed values of observed CL plus follicles $\geq 10$ mm at laparotomy.

TABLE 26. ANALYSIS OF VARIANCE FOR PHASE, ESTRADIOL VALERATE AND DAY OF INJECTION, EXPERIMENT V

| Source | d.f. | Mean square ovulation rate[a] | Mean square total stimulation[b] |
|---|---|---|---|
| Phase (P) | 1 | 0.0257[c] | 0.2491 |
| Estradiol valerate (E) | 1 | 0.2378[d] | 0.4662 |
| P x E | 1 | 0.1374 | 0.0694 |
| Day of FSH injection (D) | 2 | 0.0359 | 0.0706 |
| P x D | 2 | 0.0333 | 0.1316 |
| E x D | 2 | 0.0364 | 0.0092 |
| P x E x D | 2 | 0.4437** | 0.2417 |
| Residual | 78 | 0.0844 | 0.2380 |

** $P < .01$.

[a] Computed from transformed values of observed CL at laparotomy.

[b] Computed from transformed values of observed CL plus follicles $\geq$10 mm at laparotomy.

[c] Tabular values computed from data transformed to $\sqrt{X + 0.5}$ for analysis.

[d] $P < .10$.

TABLE 27. DISTRIBUTION OF OVULATION SITES, EXPERIMENT V, PHASES I AND II POOLED

| | | Zero CL[a] | | Single CL[a] | | Multiple CL[a,b] | | Estradiol valerate[c] | | Day of FSH[c] | | EV x day[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % | | % | | % | | $\bar{x}$ | | $\bar{x}$ | | $\bar{x}$ |
| 5.0 mg EV, FSH day | 10 | 2/15 | 13.3 | 10/15 | 66.7 | 3/15 | 20.0 | 44/45 | 0.98 | 34/30 | 1.13 | 17/15 | 1.13 |
| | 12 | 4/15 | 26.7 | 10/15 | 66.7 | 1/15 | 6.7 | | | 31/30 | 1.03 | 13/15 | 0.87 |
| | 14 | 5/15 | 33.3 | 7/15 | 46.7 | 3/15 | 20.0 | | | 33/30 | 1.10 | 14/15 | 0.93 |
| 0.0 mg EV, FSH day | 10 | 0/15 | 0.0 | 13/15 | 86.7 | 2/15 | 13.3 | 54/45 | 1.20 | | | 17/15 | 1.13 |
| | 12 | 3/15 | 20.0 | 9/15 | 60.0 | 3/15 | 20.0 | | | | | 18/15 | 1.20 |
| | 14 | 1/15 | 6.7 | 10/15 | 66.7 | 4/15 | 26.7 | | | | | 19/15 | 1.27 |
| Overall | | 15/19 | 16.7 | 59/90 | 65.5 | 16/90 | 17.8 | | | | | 98/90 | 1.09 |

[a] Nontransformed data showing frequency and percent of ovulation types observed at laparotomy.
[b] Range of multiple ovulations = 2 to 3 per heifer.
[c] Frequency of ovulation sites per group and average number of ovulations per heifer (averages represent treatment means of nontransformed data).

in these data do not permit a positive conclusion that EV injected on the second day of MGA feeding depressed ovulation rate in response to FSH. On the other hand, no justification can be made on the basis of these data to include an EV injection to heifers given 7 mg of FSH in 2 or 3 ml of 1 percent CMC on day 10, 12 or 14 of a 14-day MGA feeding period. Rao et al. (1971) reported cows treated with EV exhibited a reduced number of follicles 4.0 to 8.0 mm, no normal follicles over 8 mm and follicles over 12 mm were cystic. The cystic reaction was not indicated in the present study as only one cystic follicle was observed. However, the suggested inhibition of follicular growth by EV may explain the tendency for a reduced response to FSH which was observed when EV injections were included in the treatments used in experiment V.

The average ovulation rate resulting from single injections of 7 mg of FSH was 1.1 per heifer (computed from nontransformed data). This was distinctly lower than the 2.1 average reported by Bellows et al. (1969) which resulted from 6.25 mg of FSH given in a series of twice daily injections of 0.625 mg each. These data (experiment V, table 27) suggest that 1 percent CMC, of the type and in the ratios of FSH to CMC used in this study, did not provide a single injection method of duplicating results obtained from multiple injections of FSH as suggested by Vincent (1970). On the other hand, Vincent and Mills (1972) reported single injections of 10 mg of FSH in 3.6 or 5.0 ml of CMC resulted in an average of 2.0 and 2.3 CL, respectively. They injected FSH on day 10 to cattle which had received 5 mg of EV on day 2

of a 10-day sequence of norethandrolone injections to synchronize estrus. The CMC used by Vincent and Mills (1972) was manufactured by a different company than that used in this study and could account for some of the difference in responses observed. There are several types of CMC available (Hercules Inc., 1971).

In agreement with Bellows et al. (1969) and Laster et al. (1971), no significant correlation was shown between ovulation rate and body weight at the beginning of treatments ($r = 0.13$ in phase I, $r = 0.04$ in phase II). Correlations of total weight gain for the 28 days prior to and during treatment and ovulation rate ($r = 0.01$ in phase I, $r = 0.07$ in phase II) were also nonsignificant.

During conduction of this trial, it was observed that most heifers which had multiple ovulations (16/97, 16.5 percent) showed estrual behavior relatively soon after the end of MGA feeding (figure 1). Simple linear regression of ovulation sites on the interval from end of MGA feeding to onset of estrus detected significant ($P<.05$) regression coefficients of -.16 and -.14 ovulation sites per day post-MGA for phases I and II, respectively. These data suggest the longer the interval from termination of oral MGA to onset of estrus, the lower the ovulation rate. However, treatment effects are confounded in these comparisons and another study designed to evaluate this relationship is suggested before drawing positive conclusions.

The shorter intervals may have been due to increased follicular growth from FSH injections with the increased estrogen from these follicles hastening the onset of estrus. However, Young (1961)

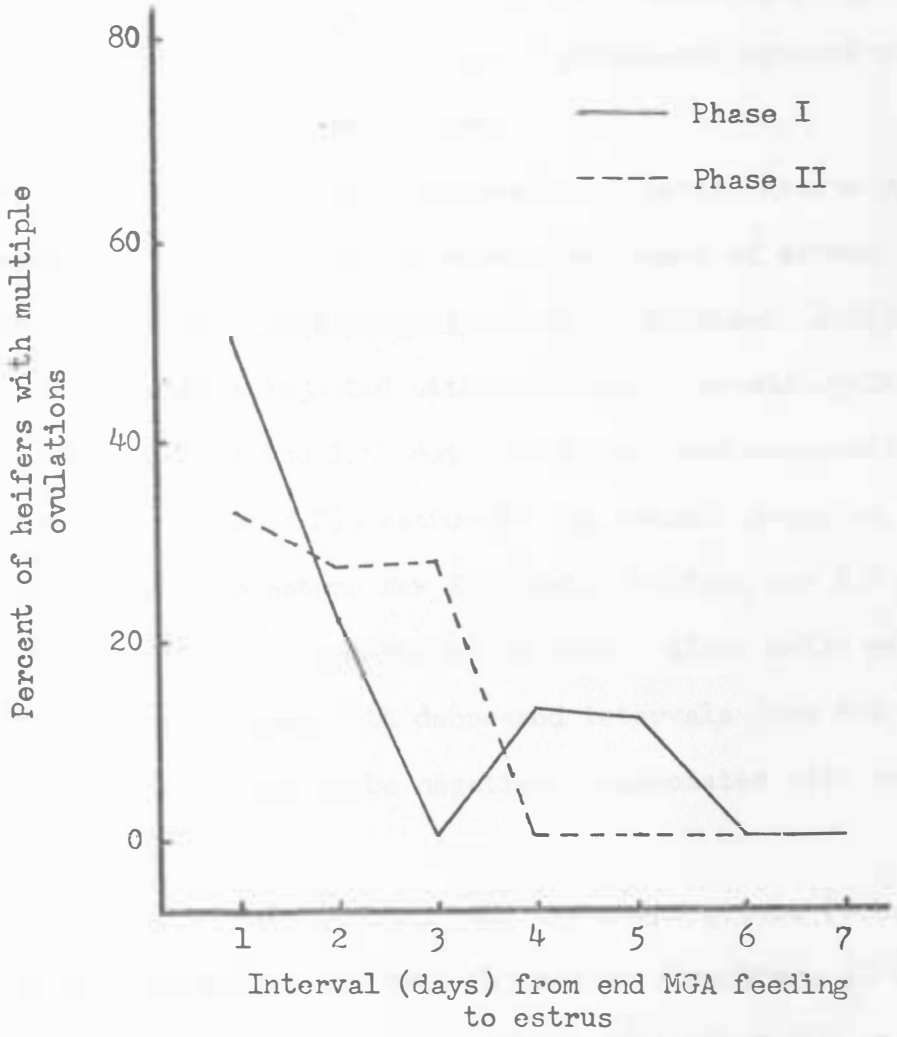


Figure 1. Relationship of multiple ovulations and MGA-to-estrus interval, experiment V.

suggested that mating behavior is associated with an apparent optimum "balance" of estrogen and progesterone, variable with species. This optimum balance may also be variable with individual animals as well as species. Hafez et al. (1965) suggested that exogenous gonadotropin (PMS) tended to increase the variability of subsequent estrous cycles rather than consistently shortening them.

There is a possibility that regression of luteal tissue by estradiol valerate might have led to an earlier onset of estrus following removal of exogenous progestin (MGA). Wiltbank (1966) reported 15 of 20 heifers injected with EV in early or mid-cycle returned to estrus before the 17th day. EV injections apparently did not affect the onset of post-MGA estrus in the present study as the mean interval from MGA to estrus for EV-treated heifers was 5.0 days as compared to 5.36 days for nontreated heifers. Also, while multiple ovulations tended to increase with decreased intervals from MGA to estrus, EV injections tended to be negatively associated with ovarian stimulation.

Conception rates were poor for all treatment groups (0 to 29 percent) and only one heifer had twin fetuses at slaughter, 31 to 44 days post-insemination. The negative effects of feeding MGA at 1 mg per head per day offers a possible explanation for low conception rates. Hill et al. (1971) reported that MGA treatments resulted in failure to show estrus, ovulation without estrus, split estrus and nonovulation. They suggested that decreased fertility following MGA could be due to a number of causes. The high incidence of anovulation

in the present study (8/48, phase I; 8/49, phase II) was probably due to the effects of MGA and agrees with the observations reported by Hill et al. (1971). On the other hand, indications of pyometria in one uterus and of remnants of regressing fetuses in 11 uteri were observed which suggests that fertilization may have taken place followed by embryonic death. Physiological stress may have been a contributing factor as Gordon et al. (1962) reported rectal palpation at six weeks resulted in a severe loss in fetuses and Schwartz and Shelby (1969) reported low conception rates in PMS treated heifers which were laparotomized following breeding.

## Experiment VI

Measures of ovarian stimulation observed in experiment V from single injections of 7 mg of FSH were relatively low which suggested larger doses and/or multiple doses. Results in experiment V also suggested a shorter interval between FSH injection and estrus. Phase I of experiment VI was designed to compare ovarian stimulation in response to two levels (7 and 10 mg) of FSH in CMC and to compare these levels administered as single or split doses (table 21). Studies conducted at this station (Section I of this thesis) indicated a ratio of 1 mg FSH to 2 ml or more of 1 percent CMC resulted in optimum ovarian stimulation in rats. The 1:2 ratio was used for all FSH injections in this experiment.

Synchronization of estrous cycles can reduce labor requirements for establishing dates from which to base injections of gonadotropins

in efforts to induce multiple ovulations in cattle. As a matter of convenience, the estrous cycles of heifers in experiment V were synchronized with oral MGA. However, results obtained in experiment V suggested a low conception rate and a relatively high incidence of nonovulating heifers (table 27) which may have been due to negative effects of MGA. Also, EV injections tended to depress rather than enhance ovulation rate in response to FSH in experiment V. On the basis of these data, estradiol valerate and synchronization treatments were omitted from experiment VI.

Analysis of variance for phase I (table 28) detected no significant differences in ovulation rate or ovarian stimulation due to treatments. Ovulation rate (computed from nontransformed data) in response to a single injection of 7 mg FSH on day 15 was nearly as great (1.5 CL per heifer) as the maximum response (1.7 CL per heifer) which was observed from 10 mg of FSH given in two injections of 5 mg each on days 15 and 17 of natural estrous cycles (table 29). This observation suggests no apparent trends in response to treatments. However, there were only 2 of 50 which did not ovulate in experiment VI as compared to 8 of 48 and 8 of 49 which were anovulatory at laparotomy in phases I and II, respectively, the previous year. It is thought that this reduction in nonovulation rate was due to the omission of MGA. The overall average of 1.32 CL represents a slight increase over the 1.09 average in experiment V. This was apparently due to a decrease in nonovulators rather than an increase in multiple ovulators (tables 27 and 29).

TABLE 28. ANALYSIS OF VARIANCE FOR EXPERIMENT VI

Phase I

| Source | d.f. | Mean square ovulation rate[a] | Mean square total stimulation[b] |
|---|---|---|---|
| Treatment | 4 | 0.0901[c] | 0.1008 |
| Residual | 45 | 0.0852 | 0.1328 |

Phase II

| Source | d.f. | Mean square fetuses[d] | Mean square CL[e] |
|---|---|---|---|
| Treatment | 4 | 0.0765[c] | 0.0868 |
| Residual | 40 | 0.0845 | 0.0560 |

[a] Computed from transformed values of observed ovulation sites at laparotomy.

[b] Computed from transformed values of observed ovulation sites plus follicles $\geq 10$ mm at laparotomy.

[c] Tabular values computed from data transformed to $\sqrt{X + 0.5}$ for analysis.

[d] Computed from transformed values of observed fetuses at laparotomy, 43 to 57 days post-insemination.

[e] Computed from transformed values of observed active CL at laparotomy, 43 to 57 days post-insemination.

TABLE 29. DISTRIBUTION OF OVULATION SITES, EXPERIMENT VI, PHASE I

| Treatment | Zero CL[a] | | Single CL[a] | | Multiple CL[a,b] | | Single or split injections[c] | | FSH level[c] | | Single or split x FSH level[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | | % | | % | | $\bar{x}$ | | $\bar{x}$ | | $\bar{x}$ |
| Control | 0/10 | 0.0 | 10/10 | 100.0 | 0/10 | 0.0 | | | | | 10/10 | 1.00 |
| 7 mg FSH, day 15 | 0/10 | 0.0 | 8/10 | 80.0 | 2/10 | 20.0 | 26/20 | 1.30 | 28/20 | 1.40 | 15/10 | 1.50 |
| 3.5 mg FSH, day 15 + 17 | 1/10 | 10.0 | 6/10 | 60.0 | 3/10 | 30.0 | 30/20 | 1.50 | | | 13/10 | 1.30 |
| 10 mg FSH, day 15 | 0/10 | 0.0 | 9/10 | 90.0 | 1/10 | 10.0 | | | 28/20 | 1.40 | 11/10 | 1.10 |
| 5 mg FSH, day 15 + 17 | 1/10 | 10.0 | 4/10 | 40.0 | 5/10 | 50.0 | | | | | 17/10 | 1.70 |
| Overall | 2/50 | 4.0 | 37/50 | 74.0 | 11/50 | 22.0 | | | | | 56/50 | 1.32 |

[a] Nontransformed data showing frequency and percent ovulation types observed at laparotomy.
[b] Range of multiple ovulations = 2 to 3 per heifer.
[c] Frequency of ovulation sites per group and average number ovulations per heifer (averages represent treatment means of nontransformed data).

As in experiment V, gross examination of the data in phase I of experiment VI suggested that the interval from FSH injection to estrus may have affected ovulation rate. There was a higher incidence of multiple ovulations regardless of treatments in those animals with the shorter estrous cycles (figure 2). Simple linear regression of ovulation sites on length of treatment estrus for phase I was negative and significant ($b = -.24$ ovulation sites per day, $P < .01$), suggesting that, when FSH injections were given closer to estrus, greater stimulation occurred. This, in turn, implies that, since the onset of estrus cannot be predicted on an individual basis (Olds and Seath, 1951), injections should probably be given later in the average estrous cycle than day 15 or day 15 plus 17. However, different treatment effects present, as discussed in experiment V, do not permit positive conclusions relative to this relationship based on these data. A similar relationship was reported by Sitton et al. (1972) in cattle treated alike. They reported a simple regression coefficient of -.58 ovulation sites per day post-treatment was shown to approach significance ($P < .10$) when total doses of 10 mg of FSH in CMC (2 mg FSH:1 ml CMC) were given in two subcutaneous injections of 5 mg each on day 16 and 18 of the estrous cycle.

Phase II of experiment VI was designed to compare ovarian responses to total doses of 10 mg of FSH in CMC given in two injections on day 15 and 17 or on days 16 and 18 of natural estrous cycles. Also, in view of possible commercial application of these treatments, groups assigned to receive FSH on days 15 and 17 or on days 16 and 18

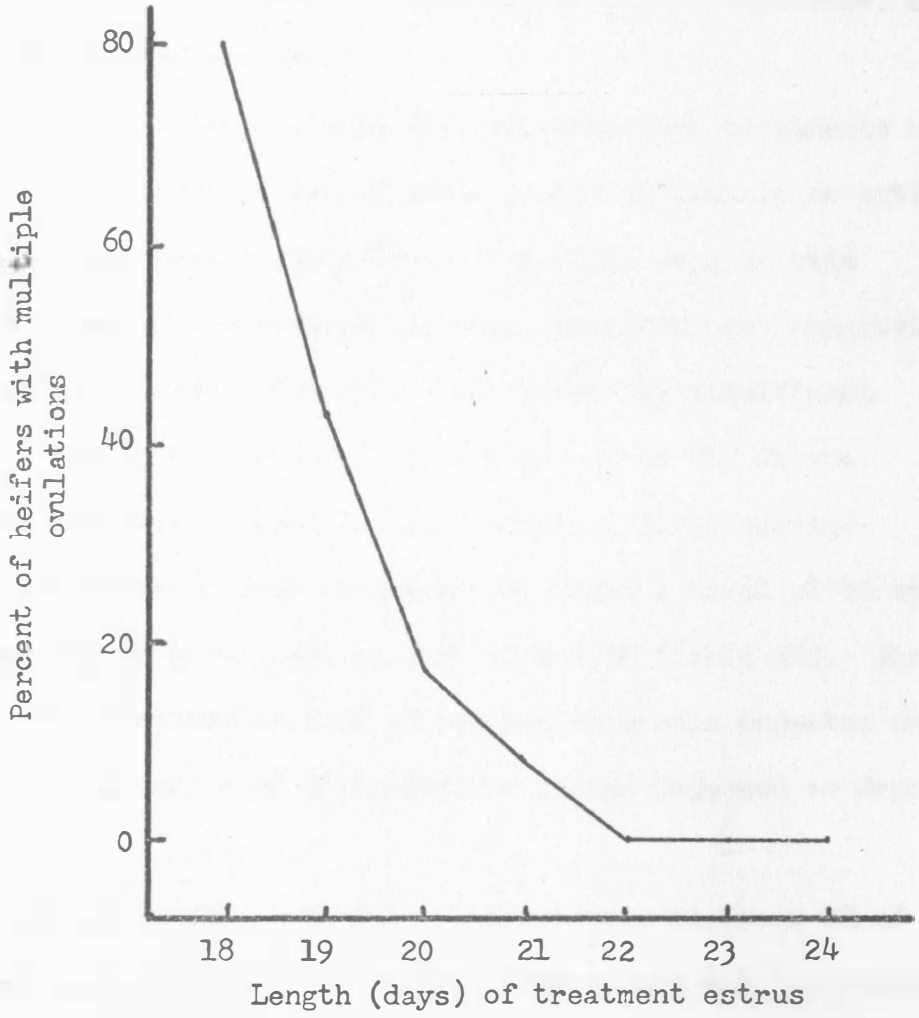


Figure 2. Relationship of multiple ovulations and length of treatment estrus, experiment VI, phase I.

were divided equally in order to study the effects of FSH injections administered during two consecutive estrous cycles (table 22). The heifers were inseminated artificially at the first estrus post-treatment and also at the estrus following the second treatment, if this second estrus was expressed.

Analysis of variance (table 28) indicated that treatments were not a significant source of variation in number of fetuses or active CL at 43 to 57 days post-insemination. Only five sets of twin fetuses were observed and nine of the uteri examined were nongravid. Although treatments were not shown as statistically significant sources of variation in number of fetuses or active CL, it was observed that all sets of twin fetuses were from first-service conceptions and occurred only in treatments where a total of 10 mg of FSH was given in two injections on days 16 and 18 (table 30). Also, multiple CL were observed in 7 of 20 heifers in groups injected on days 16 and 18 and only 1 of 20 heifers in groups injected on days 15 and 17.

The average number of CL for all treatments in phase II of experiment VI (1.2 computed from nontransformed data and including only the FSH treated groups) as well as the average from treatments on days 16 and 18 ($\bar{x} = 1.4$) were lower than expected on the basis of similar treatments reported from previous work at other stations (Bellows et al., 1969; Vincent and Mills, 1972). Sitton et al. (1972) reported an average of 2.1 ovulation sites in heifers treated with 5 mg of FSH in CMC on days 16 and 18 of natural estrous cycles. The

TABLE 30. DISTRIBUTION OF CONCEPTIONS AND NUMBER OF FETUSES, EXPERIMENT VI, PHASE II

| Measure | Control | FSH[a] day 15 + 17[b] one estrus | FSH[a] repeated[c] day 15 + 17 | FSH[a] day 16 + 18[b] one estrus | FSH[a] repeated[c] day 16 + 18 |
|---|---|---|---|---|---|
| No. cows conceiving at first estrus | 5/5 (100.0%) | 8/10 (80.0%) | 8/10 (80.0%) | 3/10 (30.0%) | 8/10 (80.0%) |
| No. cows inseminated at second estrus | 0/5 ( 0.0%) | 2/10 (20.0%) | 2/10 (20.0%) | 7/10 (70.0%) | 2/10 (20.0%) |
| No. cows carrying only one fetus and pregnant to first service | 5/5 (100.0%) | 6/10 (60.0%) | 6/10 (60.0%) | 0/10 ( 0.0%) | 4/10 (40.0%) |
| No. cows carrying only one fetus and pregnant to second service | 0/5 ( 0.0%) | 2/10 (20.0%) | 2/10 (20.0%) | 4/10 (40.0%) | 2/10 (20.0%) |
| No. cows nonpregnant from first service but second estrus not detected | 0/5 ( 0.0%) | 2/10 (20.0%) | 2/10 (20.0%) | 1/10 (10.0%) | 1/10 (10.0%) |
| No. cows nonpregnant from second service | 0/5 ( 0.0%) | 0/10 ( 0.0%) | 0/10 ( 0.0%) | 3/10 (30.0%) | 0/10 ( 0.0%) |
| No. cows with multiple fetuses from first service | 0/5 ( 0.0%) | 0/10 ( 0.0%) | 0/10 ( 0.0%) | 2/10 (20.0%) | 3/10 (30.0%) |
| No. cows with multiple fetuses from second service | 0/5 ( 0.0%) | 0/10 ( 0.0%) | 0/10 ( 0.0%) | 0/10 ( 0.0%) | 0/10 ( 0.0%) |

[a] Total dose of FSH = 10 mg injected in two doses of 5 mg each on days cited (day 0 = observed onset of estrus).

[b] FSH injections at first estrus, volumetric equivalents of CMC only injected at following estrus.

[c] FSH injections repeated at second estrus (day 0 = second observed onset of estrus, with insemination on day 1).

procedure they reported was identical to the day 16 and 18 sequence used in the present study except that ratios of FSH to CMC were different (1 mg FSH:2 ml CMC in this study vs. 2 mg FSH:1 ml CMC).

A refractory reaction to FSH in the heifers which were retreated after an interval of 12 weeks offers a possible explanation for the lower than expected ovulation rate observed. Refractoriness was suggested from the observation that only two of the eight multiple CL observed and none of the multiple fetuses occurred in response to second cycle injections of FSH. Jainudeen et al. (1966) reported that the same doses of PMS repeated at subsequent estrous cycles in cattle failed to stimulate ovaries, but an increased dose of PMS after a delay of 5 to 7 months resulted in ovarian responses similar to those of the initial treatment.

## Summary

A total of 99 yearling, commercial beef heifers was used in two trials conducted during consecutive summers (1970 and 1971) to study ovarian stimulation in response to follicle stimulating hormone (FSH) carried in carboxymethylcellulose (CMC). Each trial was conducted in two phases to provide two data collections from each group of experimental animals.

In the first experiment, 48 heifers were fed 1 mg melengesterol acetate (MGA) per head per day for 14 days to synchronize estrus. One-half of the heifers were injected with 5 mg of estradiol valerate (EV) on day 2 of MGA feeding to induce regression of luteal tissue. Each heifer received a single injection of 7 mg FSH in 1 percent CMC on either day 10, 12 or 14 of MGA feeding to induce multiple ovulation. In phase II, the same treatments were repeated with the exception that a control group was included and all heifers were inseminated artificially at the first post-treatment estrus. Measures of ovarian stimulation were obtained by high lumbar laparotomy at 72 to 96 hours following post-treatment estrus in both phases and conception rate was determined by examination of uteri recovered at slaughter 31 to 44 days post-insemination.

Day of injection of FSH was not a significant source of variation in ovulation rate or total ovarian stimulation (ovulation sites plus follicles $\geq$10 mm). EV injections tended to depress ovulation rate ($P < .10$), but a significant phase x EV x day of injection interaction ($P < .01$) limited positive conclusions concerning

this effect. Conception rates were low for all treatment groups (0 to 29 percent), and the incidence of anovulatory heifers was relatively high (8/48 and 8/49 for phases I and II, respectively).

There was no indication of a relationship between body weight or rate of gain (28 day period prior to and during treatment) and ovarian stimulation. However, significant ($P < .05$) regression coefficients (-.16 and -.14 ovulation sites per day post-MGA) suggested that the longer the interval from termination of oral MGA to onset of estrus, the lower the ovulation rate. Treatment effects were confounded in this relationship and further study was suggested prior to drawing positive conclusions.

Phase I of the second trial involved 50 yearling heifers and was designed to compare ovarian stimulation in response to two levels (7 and 10 mg) of FSH in CMC and to compare these levels injected as single or split doses on day 15 or days 15 and 17 of natural estrous cycles.

No significant differences in ovulation rate or total ovarian stimulation were detected due to treatment and no trends in response to treatments were suggested by the data. The number of nonovulating heifers was lower (2/50) than in the previous trial. In agreement with the results in the first trial, there was an indication that, when FSH was injected closer to estrus, greater ovarian stimulation occurred.

Phase II of the second trial was designed to study ovarian response to total doses of 10 mg FSH in CMC given in two injections

on days 15 and 17 or days 16 and 18 of natural estrous cycles and to study the effects of these same treatments applied during two consecutive cycles. All heifers were inseminated artificially at the first and also second post-treatment estrus, if expressed. Parameters of response were number of fetuses and active corpora lutea (CL) observed at laparotomy 43 to 57 days following the last insemination.

Treatments were not a significant source of variation in number of fetuses or active CL. Ovarian stimulation was low with five sets of twin fetuses and nine nongravid uteri observed in the 45 heifers involved. However, all twin fetuses were from first service conceptions and occurred only in treatment groups where FSH was injected on days 16 and 18. Also, multiple CL were observed in 7 of 20 heifers in groups which received FSH on days 16 and 18 and 1 of 20 heifers in groups injected on days 15 and 17.

A refractory reaction to FSH was suggested in this second experiment as only two of eight multiple CL observed and none of the multiple fetuses resulted from second cycle injections of FSH.

# GENERAL DISCUSSION

Bio-assay procedures, involving immature female rats, were employed to demonstrate that ovarian and uterine growth in response to exogenous follicle stimulating hormone (FSH) was greater when carboxymethylcellulose (CMC) was used as the carrier than when the same level of FSH was injected with physiological saline. It was demonstrated further that the apparent optimum ratio of FSH to 1 percent CMC in the rat was 1 mg FSH to 2 ml or more of CMC. These data tend to support the suggestion by Vincent (1970) that CMC may reduce the number of injections of FSH required to achieve controlled multiple ovulations in beef cattle by prolonging systemic absorption of FSH injections. However, these same data do not permit conclusions that the apparent enhancement of FSH by CMC in the rat was due to a prolonged absorption effect per se. Also, the results of these experiments as designed and conducted do not permit conclusions relative to the mechanisms by which the enhancing effects of CMC were exerted. These mechanisms may involve the colloidal and/or the ion exchange properties of CMC.

CMC is available commercially with several different degrees of substitution (number of carboxymethyl groups per anhydroglucose unit) and in several viscosities (Hercules Inc., 1971). Further study is suggested to determine whether the relationship of CMC to FSH is physical and/or chemical which could, in turn, lead to determination of an optimum type of CMC as well as ratio of CMC to FSH.

The effects of level and day of injection of FSH (carried in CMC) were studied in a series of experiments with synchronized and nonsynchronized beef heifers. The negative effects of MGA reported by Hill et al. (1971) were reaffirmed in this study. The need for a good synchronization compound seems most imperative.

The dose and day of injection treatment combination used in these experiments did not affect ovarian stimulation significantly. Ovulation rate was lower than that reported by Vincent and Mills (1972) from treatments similar to those used in the present study. However, the CMC used was manufactured by different companies and may have had considerably different properties. Further study with closer attention to type of CMC used seems justified.

The data of this study indicated that CMC, of the type and in the ratios used, did not provide a method for greatly reducing the number of injections required to cause controlled multiple ovulation in cattle. One or two injections of FSH with CMC as the carrier did not stimulate ovulation rate as strongly as similar total dosages of FSH given twice daily over a period of 5 days (Bellows et al., 1969). However, the enhancing effect of CMC on ovarian and uterine growth observed in the rat suggests additional study before discarding this material as a possible useful carrier for FSH.

# GENERAL SUMMARY

This study was conducted in search of a practical procedure of administering follicle stimulating hormone (FSH) to achieve controlled multiple ovulation in beef cattle.

During the winter and spring of 1970 and 1971, a series of experiments were conducted to compare physiological saline (PSS) and carboxymethylcellulose (CMC) as carriers for FSH and to determine an optimum ratio of FSH to CMC. Constant individual dosages of 0.5 mg FSH were injected into a total of 384 immature female rats. Treatments included single injections of FSH in PSS, FSH in 1 percent CMC at ratios (mg FSH:ml CMC) of 1:3, 1:2, 1:1, 2:1, 3:1, 5:1 and FSH in 2 percent CMC at a ratio of 1:1. Parameters of primary and secondary response were the combined weight of left and right ovaries and the weight of the uterus, respectively, at 24, 48, 72 and 96 hours post-injection.

It was demonstrated that ovarian and uterine growth resulting from FSH in CMC was greater than from the same quantity of FSH in PSS. The apparent optimum ratio of FSH to CMC (of the type used in this study) was 1 mg FSH in 2 ml or more of 1 percent CMC. The mechanisms by which CMC tended to enhance the activity of FSH could not be determined from these experiments as designed and conducted.

During the summers of 1970 and 1971, CMC was used as the carrier for FSH injections to study the effects of dosage and day of FSH injection in cattle. A total of 99 beef heifers were used in two experiments. Each trial was conducted in two phases to permit maximum

utilization of experimental animals by administering treatments early in the summer, allowing a period for recovery, and using the same animals again in early fall.

Treatments studied the first summer were 7 mg FSH in 1 percent CMC injected on days 10, 12 or 14 of a 14-day feeding of melengesterol acetate (MGA) to synchronize estrus. The heifers were started on MGA without regard to stage of estrous cycles. One-half of each of these treatment groups had received injections of 5.0 mg of estradiol valerate (EV) on day 2 of MGA feeding to induce regression of luteal tissue. Degree of ovarian stimulation was determined by high lumbar laparotomy at 72 to 96 hours following post-treatment estrus.

Day of FSH injection did not affect the number of ovulation sites or total ovarian stimulation (ovulation sites plus follicles $\geq$10 mm) significantly. EV injections tended to reduce ovulation rate ($P < .10$) in response to exogenous FSH. However, a significant phase x EV x day of injection interaction ($P < .01$) did not permit positive conclusions relative to the apparent negative effect of EV. Average ovulation rate for all treatments was low (1.1 ovulation sites per heifer). Conception rates from one post-treatment artificial insemination were also low for all treatment groups (0 to 29 percent at slaughter, 31 to 44 days post-insemination in phase II). The frequency of anovulatory heifers was relatively high (8/48 and 8/49 for phases I and II, respectively). The incidence of multiple ovulations tended to be higher in heifers with shorter MGA-to-estrus intervals. Hence, it was suggested that the interval from termination

of MGA feeding to estrus was negatively associated with ovulation rate.

Phase I treatments in the second experiment were total doses of 7 or 10 mg of FSH in CMC (1 mg FSH:2 ml 1 percent CMC) injected as a single dose on day 15 or two half doses on days 15 and 17 of natural estrous cycles. Phase II treatments were 5 mg of FSH injected on days 15 and 17 or on days 16 and 18 (total of 10 mg) of natural cycles. The same treatments were administered to one-half of each group during one subsequent cycle. All heifers in phase II were inseminated artificially at the first post-treatment estrus and again at the second estrus, if it was expressed. Ovarian stimulation in response to treatments was determined by laparotomy at 72 to 96 hours following post-treatment estrus in phase I and at 43 to 57 days following the last insemination in phase II.

Differences due to treatments were not significant in either phase and the tendency for ovarian stimulation to be greater in heifers with shorter treatment-to-estrus intervals observed in the previous experiment was repeated in phase I.

Average ovulation rate for all heifers receiving FSH in phase I was 1.4 per heifer. Five sets of twin fetuses and nine nongravid uteri were observed in 45 heifers in phase II. However, all twin fetuses were from first service conceptions and occurred only in heifers receiving FSH injections on days 16 and 18. Also, multiple CL were observed in 7 of 20 heifers in groups which received FSH on

days 16 and 18 and 1 of 20 heifers in groups injected on days 15 and 17.

A refractory reaction to FSH was suggested as only two of eight multiple CL observed and none of the multiple fetuses resulted from second cycle injections of FSH.

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